# Transcriptional Regulation and Functions of the *Drosophila paired* Gene

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Sreehari Kalvakuri

aus der

Republik Indien

Promotionskomitee

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Zürich 2009

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## Summary

The *paired* (*prd*) gene as well as the *gooseberry* (*gsb*) and *gooseberry neuro* (*gsbn*) genes of *Drosophila* are the founding members of the Pax gene family and part of the Pax-3/7 subfamily. As shown previously in this lab, *prd* encodes a transcription factor containing two DNA binding domains, a paired-domain and an extended *prd*-type homeodomain, in its N-terminal half and a 21-amino acid His-Pro or PRD repeat near its C-terminus, which serves as the major activation domain. Although *prd*, *gsb*, *gsbn*, and *Pax3* have divergent C-terminal sequences and exhibit distinct functions, they share highly conserved DNA binding domains in the N-terminal half and are probably evolved by gene duplication from the same ancestral gene. In combination with other pair-rule genes, *prd* specifies the positional information along the antero-posterior axis and activates the segment polarity genes, including *gsb*, *wingless*, and *engrailed*. In addition to its embryonic segmentation function, *prd* is necessary for post-embryonic viability and male fertility. Two Prd homologs, the *Drosophila* Gsb and murine Pax3 proteins are able to substitute for some of the Prd functions when they are placed under the control of the complete *cis*-regulatory region of *prd*, which demonstrates that the *cis*-regulatory elements play a dominant role during evolution.

In this study, we investigated the *cis*-regulatory elements of *prd* in great detail, using a series of partial rescue transgenes and reporter transgenes. The results show that there are at least three enhancer regions in the *prd* locus, which can produce a striped pattern independent of each other. Our previous results showed that multiple *cis*-regulatory elements in the upstream region produce a striped pattern in the embryo. Here, we could demonstrate that the downstream region and intron sequences also produce a striped pattern. Rescue experiments with transgenes affording partial rescue uncovered the haplo-insufficiency of upstream enhancers for survival and adult cuticle functions of *prd*. This haplo-insufficiency of upstream enhancers is rescued completely by a single copy of a *prd* transgene under the sole control of the downstream enhancers. Investigation of these enhancers in detail uncovered a partial redundancy in the downstream control region required for survival and adult cuticle functions of *prd*.

Investigation of the male fertility function has previously revealed that *prd* is necessary for the development of accessory glands by promoting cell division and for the transcriptional regulation of genes essential for the function of the accessory glands. Downstream sequences of *prd* are necessary for the development of accessory glands. Here, we show that the upstream sequences also control *prd* expression in the adult accessory glands. The enhancer responsible for *prd* expression in the accessory glands has been mapped to a 590 bp fragment in the upstream region and shown to be crucial for the male fertility function. Interestingly, *prd* mutants rescued by a transgene, regulated by the entire upstream region but lacking the accessory gland enhancer, and by a transgene under control of the downstream region of *prd* develop accessory glands, but the glands are small in size and show reduced expression of Prd in freshly eclosed males. These glands gradually go through morphological changes that end with the complete collapse of the gland's lumen while Prd protein levels are strongly elevated in virgin males older than 5 days. This persistent and strong expression of Prd might result from a failure in feedback regulation that is observed in the accessory glands of wild-type virgin males: *prd* transcription in accessory glands is repressed when the gland's lumen is filled with secretory proteins, and this repression is relieved when the gland's content is emptied during mating.

Our earlier results suggest that *prd* is required for proper segmentation of the adult abdomen. Conditional knockout of *prd* in this study corroborated a crucial role of *prd* in adult cuticle segmentation. The enhancer partly responsible for the adult segmentation function has been identified in the downstream region of *prd*. It functions in the histoblasts to control the development of abdominal segments. This is evident from experiments demonstrating that the removal of *prd* function in the histoblasts is sufficient to produce the adult segmentation phenotype. Finally, we could identify the enhancers that control *prd* expression in the dorsal spot of embryos, in the ventral gustatory organ of the larvae, and in the trochanter segment of the legs.

In summary, several novel enhancers for newly discovered functions and/or expression patterns were characterized, more than initially expected.

# Zusammenfassung

Das *paired* (*prd*) Gen sowie die beiden Gene *gooseberry* (*gsb*) und *gooseberry neuro* (*gsbn*) von *Drosophila* sind die Gründungsmitglieder der *Pax*-Gen-Familie und ein Teil der Pax-3/7 Unterfamilie. Wie früher in diesem Labor gezeigt wurde, kodiert *prd* für einen Transkriptionsfaktor mit zwei DNA-bindenden Domänen, einer Paireddomäne und einer erweiterten Homeodomäne vom *prd*-Typ, in seiner N-terminalen Hälfte und gegen das C-terminale Ende mit einem 21 Aminosäure langen His-Pro oder PRD Repeat, der als starke Aktivierungsdomäne dient. Obwohl *prd*, *gsb*, *gsbn* und *Pax3* unterschiedliche C-terminale Sequenzen und verschiedene Funktionen haben, besitzen sie hochkonservierte DNA-bindende Domänen in der N-terminalen Hälfte und entwickelten sich wahrscheinlich durch Gen-Duplikation aus dem gleichen Urgen. Zusammen mit anderen Paarregelgenen wirkt *prd* im Embryo bei der Etablierung der Position auf der von vorne nach hinten verlaufenden Achse mit, indem es die Transkription von Segment-Polaritätsgenen, einschliesslich *gsb*, *wingless* und *engrailed* aktiviert. Zusätzlich zu dieser Funktion während der embryonalen Segmentierung ist *prd* wichtig für die post-embryonale Entwicklung und für die Fruchtbarkeit von Männchen. Die schon erwähnten homologen Proteine, Gsb von *Drosophila* und Pax3 der Maus, sind in der Lage einige dieser Funktionen von *prd* auszuüben, wenn sie unter der Kontrolle der vollständigen *cis*-regulatorischen Region des *prd* Gens stehen. Daraus lässt sich schliessen, dass die *cis*-regulatorischen Elemente eine dominante Rolle während der Evolution spielen.

In dieser Forschungsarbeit analysierten wir die *cis*-regulatorischen Elemente von *prd* im Detail anhand einer Reihe von *prd*-Transgenen, welche die *prd* Mutanten nur partiell retten können und von *prd*-Reportergenen, die nur einen Teil der Expression von *prd* zeigen. Die Ergebnisse zeigen, dass es mindestens drei „Enhancer"-Regionen im *prd* Lokus gibt, die unabhängig von einander ein Streifen-Muster im Embryo bilden können. Unsere früheren Ergebnisse zeigten, dass mehrere *cis*-regulatorische Elemente, die vor dem *prd* Gen liegen, ein Streifen-Muster bilden können. Zu unserer Überraschung fanden wir jetzt, dass sowohl das Intron des *prd* Gens wie auch die Region hinter dem Gen dies unabhängig auch können. Nicht nur für das Expressionsmuster sondern auch für die Segmentierungsfunktion zeigen diese drei *prd* Enhancerregionen eine teilweise Redundanz. Die Haploinsuffizienz der

‘upstream’-Enhancer von *prd* für das postembryonale Überleben und die Segmentierung im adulten Abdomen wurde mit *prd*-Transgenen gezeigt, welche die *prd* Mutanten nur teilweise retten. Diese Haploinsuffizienz der ‘upstream’-Enhancer kann durch nur ein einziges *prd*-Transgen unter der ausschliesslichen Kontrolle von ‘downstream’-Enhancern vollständig gerettet werden.

Die Untersuchung der Funktion von *prd* für die Fruchtbarkeit der Männchen hat früher ergeben, dass *prd* für die Entwicklung der akzessorischen Geschlechtsdrüse, die der Prostata von Säugetieren entspricht, unverzichtbar ist, da es dort die Zellteilung fördert und wichtige Gene für die Funktion der Drüse reguliert. Dabei wurde auch gezeigt, dass ‘downstream’-Sequenzen von *prd* für die Entwicklung der akzessorischen Geschlechtsdrüse unentbehrlich sind. Zu unserer Überraschung sind jedoch auch die ‘upstream’-Sequenzen wichtig für die korrekte Expression von *prd* in akzessorischen Geschlechtsdrüsen und dadurch für die Fruchtbarkeit der erwachsenen Männchen. Frisch geschlüpfte Männchen, die ohne den veranwortlichen 590-bp grossen ‘upstream’-Enhancer gerettet wurden, haben kleinere akzessorische Geschlechtsdrüsen mit verminderter Expression von *prd.* Mit dem Alter bricht das Lumen der Drüse vollständig zusammen und *prd* wird dann sogar stark überexprimiert. In der missgebildeten Drüse kommt diese Überexpression möglicherweise durch diesselbe Rückkoppelungs-Regulation zustande, die in der gesunden Drüse durch die Entleerung der Drüse während der Kopulierung beobachtet wurde. Denn *prd* wird in zölibatär gehaltenen Männchen fast vollständig reprimiert, höchstwahrscheinlich weil die Drüse gefüllt bleibt und kein Nachschub an sekretorischen Proteinen nötig ist. Erst die Entleerung der Drüse während der Kopulation führt zur Reaktivierung der *prd* Transkription.

Aufgrund von Experimenten, die in diesem Labor durchgeführt wurden, bestand der Verdacht, dass *prd* auch für die ordnungsgemäße Segmentierung während der Entwicklung des erwachsenen Abdomens sorgt. Die vollständig fusionierten abdominalen Segmente von Fliegen, bei denen fast alle postembryonale *prd*-Expression ausgeschaltet wurde, lassen jetzt keine Zweifel mehr offen. Der dafür teilweise verantwortliche Enhancer wurde hinter dem Gen identifiziert und funktioniert in Histoblasten während des Puppenstadiums, was gezeigt werden konnte, als *prd* nur in diesen Zellen ausgeschaltet wurde. Schliesslich konnten wir die

Enhancers identifizieren, welche die *prd*-Expression im dorsalen Fleck der Embryos, im ventralen Geschmacksorgan der Larven und im Trochanter-Segment der Beine der Puppen und erwachenen Fliegen steuern.

Zusammenfassend wurde eine Vielzahl von definierbaren Enhancern für neu entdeckte Funktionen und/oder Expressionsmuster von *prd* nachgewiesen, mehr als anfänglich erwartet.

# Chapter 1

# General introduction

One of the most fundamental and exciting questions in biology is how a multicellular organism, like a mouse or man, develops from a single cell, the fertilized egg. This process requires both the determination of different cell types and the organization of these cell types into elaborate patterns. The formation of such patterns depends on the proper establishment of positional information throughout the embryo, but the molecular nature of such information remained poorly understood for a long time. In *Drosophila*, the fertilized egg develops during embryogenesis into a segmented, fully differentiated first instar larva over a period of 22 hours at 25°C. In a pioneering genetic screen, Nüsslein-Volhard and Wieschaus identified about 50 genes, the maternal coordinate genes and three classes of zygotic segmentation genes – the gap, pair-rule, and segment-polarity genes – that control early embryonic development of *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980). These genes interact in a hierarchical network to progressively define position with increasing precision along the antero-posterior (AP) axis of the embryo (Fig. 1; Harding et al., 1986; Macdonald et al., 1986; Ingham et al., 1988; Baumgartner and Noll, 1990). The availability of sophisticated genetic tools in *Drosophila* was instrumental in the elucidation of this process of segmentation, which is one of the best understood developmental processes in metazoans (Kornberg and Tabata, 1993; Lawrence and Sampedro, 1993; Patel, 1994; Levine, 2008).

Shortly after fertilization, the Bicoid, Hunchback, and Nanos proteins are translated from maternal mRNAs and distributed in concentration gradients along the AP axis in the early *Drosophila* embryo (Fig. 1; Nüsslein-Volhard et al., 1987; Hoch and Jäckle, 1993). Contrary to the general opinion, the Bicoid protein gradient is generated by an mRNA gradient, preformed in the early embryo (Spirov et al., 2009). These maternal gene products acts as morphogens in the syncytial blastoderm to activate the gap genes in overlapping domains, each of which spanning several segments in the embryo (Fig. 1; Hülskamp et al., 1990; Kornberg and Tabata, 1993). Once activated, the gap gene products, which are mostly zinc finger containing transcription factors, regulate each other by mutual repression to form broad and partially overlapping concentration gradients (Pankratz and Jäckle, 1990; Hülskamp et al., 1990; Hülskamp and Tautz, 1991; Rivera-Pomar and Jäckle, 1996.). These

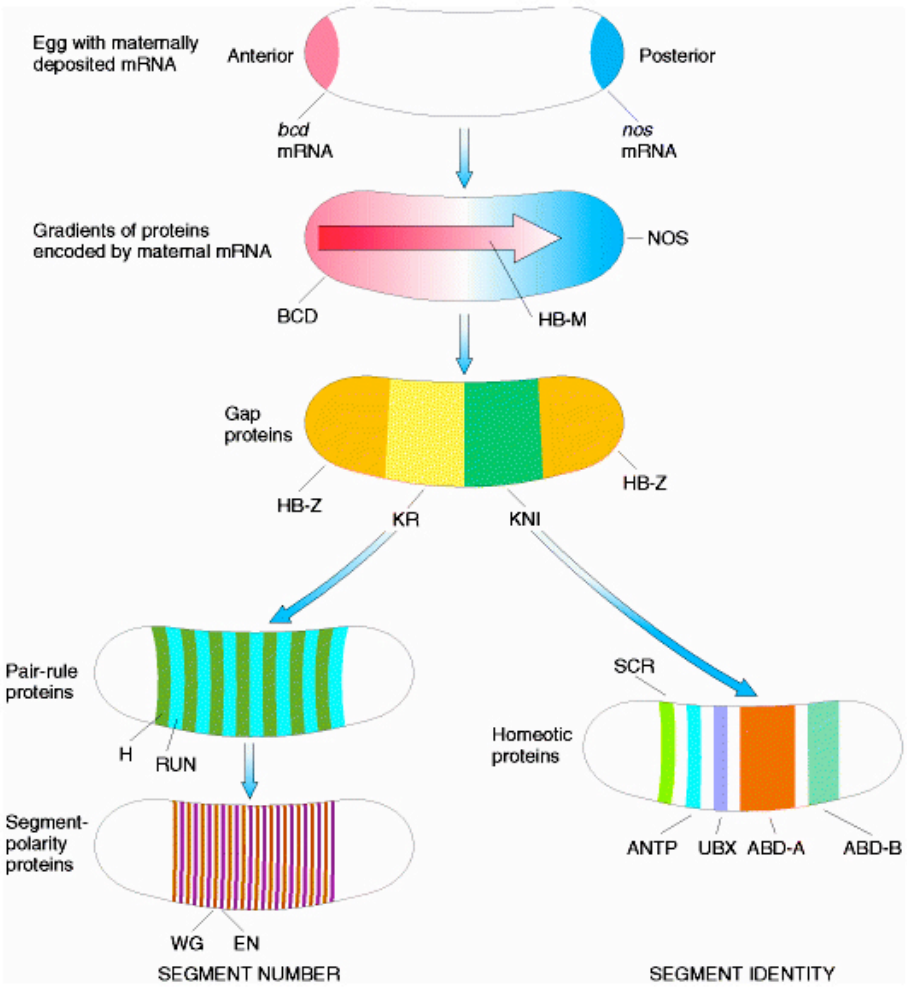


**Figure 1**. **A depiction of the hierarchical cascade that activates the elements forming the A–P segmentation pattern in *Drosophila.*** The maternally derived *bcd* and *nos* mRNAs are located at the anterior and posterior poles, respectively. However, immediately after fertilization, the *bcd* mRNA is distributed in an evolving gradient that reaches its final shape by nuclear cycle 10 and disappears rapidly during nuclear cycle 14 (Frigerio et al., 1986; Spirov et al., 2009). It is this *bcd* mRNA gradient that dictates the Bicoid protein gradient (Spirov et al., 2009). The gap genes, which are the A–P cardinal genes, are activated in different parts of the embryo in response to the anterior–posterior gradients of the two factors BCD and HB-M. The correct number of segments is determined by activation of the pair-rule genes in a zebra-stripe pattern in response to the gap gene–encoded transcription factors. The segment-polarity genes are then activated in response to the activities of the several pair-rule proteins, leading to further refinement of the organization within each segment. The correct identities of the segments are determined by expression of the homeotic genes due to direct regulation by the transcription factors encoded by the gap genes. Adapted from Kornberg and Tabata, 1993.

gap protein gradients (Gaul and Jäckle, 1990; Small et al., 1991; Small et al., 1992), in combination with the maternal protein gradients (Jäckle et al., 1992; La Rosée et al., 1997), serve as position-specific transcriptional activators/repressors to regulate the expression pattern of the pair-rule genes (Warrior et al., 1990; Small et al., 1991; Häder et al., 1998). The different transcription factors encoded by gap and maternal genes interact directly with the individual enhancers of the pair-rule genes to generate their expression patterns displaying a double-segment periodicity (Fig. 1; Harding et al., 1989; Stanojevic et al., 1991; Klingler et al., 1993; Yu and Pick, 1995; Arnosti et al., 1996). The activation of segment-polarity genes is regulated by pair-rule genes, most of which also encode transcription factors (Fig. 1; Li et al., 1993; Bouchard et al., 2000: Swantek and Gergen, 2004).

In maternal coordinate mutants, the anterior and/or posterior segments of the larval cuticle are missing. In gap mutants, several adjacent segments are missing and thus exhibit large gaps in their larval cuticular pattern (Nüsslein-Volhard and Wieschaus, 1980). Pair-rule mutants have only half the number of segments, as they are missing the equivalent of a segment in every other segment but show different phases of segment deletions (Nüsslein-Volhard and Wieschaus, 1980). In segment-polarity mutants, within each segment a part is missing and the remaining part duplicated in an apparent mirror symmetry (Nüsslein-Volhard and Wieschaus, 1980). From the analysis of gene expression patterns in mutants deficient for one or more segmentation genes, fate maps were constructed that gave an initial guide to possible regulatory interactions among these genes (Harding et al., 1986; Baumgartner et al., 1990). Regions of activity of specific genes and their proteins are now known (Fig. 1; Baumgartner et al., 1990; Gutjahr et al., 1993), and this has corroborated some of the models on gene activity and regulation (Hunding, 1993). Thus, the four classes of segmentation genes together with the homeotic genes regulate the fate of the cells in the early embryo precisely according to their position along the anterior-posterior axis. In addition, their fate is determined by positional information along the dorsoventral axis in a similar fashion (Stathopoulos et al., 2002), which is, however, not the subject of this dissertation.

It is well established that each segmentation gene has extended regulatory sequences containing multiple target sites for the regulation of its activity (Gutjahr et al., 1994; Arnosti et al., 1996). *Cis*-regulatory elements can be located upstream, downstream, or even within the gene they control (Gutjahr et al., 1994). Regulation of gene expression is achieved at the transcriptional level through binding of segmentation gene products to the *cis*-regulatory

elements of downstream genes (Small et al., 1991). Enhancer sensitivity depends on the affinity, number, and arrangement of binding sites. There is a separate enhancer controlling the expression of each stripe of gene expression in some of the pair-rule genes (Small et al., 1992). For example, in the upstream region of the *even-skipped* (*eve*) gene there are separate regulatory elements, each controlling expression in specific stripes (Stanojevic et al., 1991; Arnosti et al., 1996).

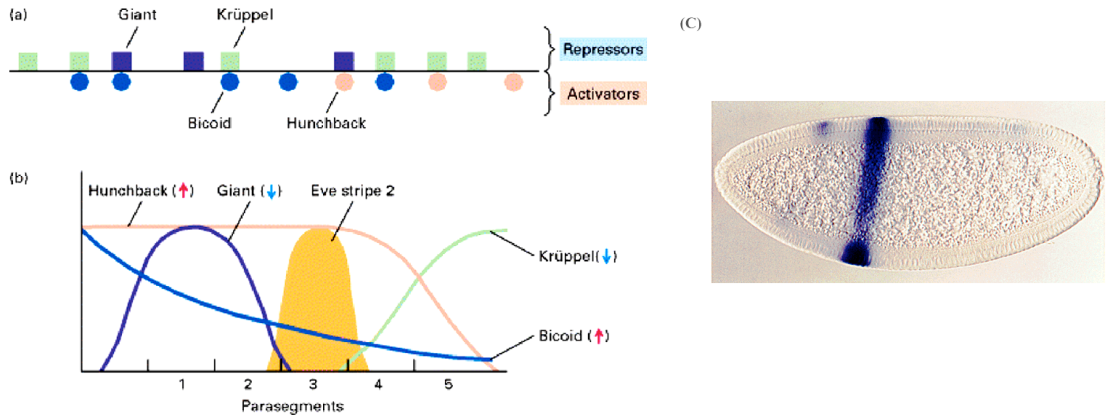


**Figure 2**. Expression of the Even-skipped (Eve) stripe-2 in the *Drosophila* embryo. (a) Diagram of the 815-bp regulatory region controlling transcription of the pair-rule gene, *eve.* This region contains binding sites for Bicoid and Hunchback proteins, which activate transcription of *eve,* and for Giant and Krüppel proteins, which repress transcription. (b) Concentration gradients of Eve stripe 2 and of the four proteins that regulate its expression. The coordinated effect of the two repressors and two activators determine the precise boundaries of the second anterior Eve stripe. (c) Expression of Lac Z under the control of eve-stripe-2 enhancer. Adapted from Small et al., 1992.

Thus, the stripe-2 enhancer controls the expression of only stripe-2 (Fig. 2). Multiple interactions of repressor proteins with the stripe-2 enhancer are directly responsible for setting the spatial limits of stripe-2 of *eve* expression (Fig. 2; Small et al., 1992). These repressor proteins interfere with the cooperative binding of activators, which recognize target sites that mostly overlap with the repressor binding sites (Fig. 2). Thus, repressors play a dominant role in the regulation of stripe 2 expression of *eve*.

### The *Drosophila paired* gene

The *paired* (*prd*) gene, initially characterized as a pair-rule segmentation gene (Nüsslein-Volhard and Wieschaus, 1980), encodes a transcriptional factor containing a paired domain (PD) and an extended *prd*-type homeodomain (HD) in its N-terminal half (Bopp et al., 1986)

and a 21 amino acid His-Pro or PRD repeat near its C-terminal end (Frigerio et al., 1986) that serves as the principal activation domain (Xue et al., 2001). Prd protein is required for the activation of segment-polarity genes, proper development of larval cuticle, post-embryonic viability and male fertility (Xue et al., 2001; Xue and Noll, 2002).

Prd is an interesting transcription factor because it contains two DNA binding domains in its N-terminal portion, a PD and a HD (Bopp et al., 1986), while most transcription factors contain only one DNA binding domain. Two of the five classes of the Pax gene family encode, in addition to the paired doamin, a homeodomain (Noll, 1993). In vitro experiments suggest that these two DNA binding domains can function either independently or cooperatively when present in the same protein (Treisman et al., 1991; Underhill et al., 1995; Jun and Desplan, 1996). However, in vivo results demonstrate that both the PD and HD of Prd have to be present in the same molecule for the activation of segment-polarity genes, larval cuticle formation, viability, and male fertility (Xue et al., 2001). In addition to its His-Pro repeat that serves as the major activation domain required for all functions of Prd, the C-terminal part of Prd contains at least two other functionally important motives. One of them, required for the activation of segment-polarity genes and proper development of larval cuticle, is present in the C-terminal moieties of both Gsb and Pax3 (Xue et al., 2001). The second motif, necessary for the post-embryonic viability function of Prd, is present only in the C-terminal half of Gsb but not in Pax3 (Xue et al., 2001). The male fertility function of Prd, which cannot be replaced by its evolutionary alleles, *prd*-Gsb and *prd*-Pax3, requires the conserved N-terminal DNA binding domains rather than the divergent C-terminal portion of Prd (Xue et al., 2001).

Prd expression is initially detectable in a single broad anterior stripe at the end of the 13th nuclear division of syncytial blastoderm (Gutjahr et al., 1993). By mid-cellularization, Prd expression is seen in an anterior dorsal patch and a characterstic pair-rule pattern of seven stripes. By cellular blastoderm, this pair-rule pattern is converted into 14 segment-polarity-like stripes spanning each parasegmental boundary. During germ band extension, Prd expression decreases and gradually disappears in the epidermal stripes but later accumulates in the gnathal segments of the prospective head region and in a few specific cells of the central nervous system (Gutjahr et al., 1993). In combination with other pair-rule genes, *prd* specifies positional information in every other segment and activates the segment-polarity genes, including *gooseberry* (*gsb*), *wingless* (*wg*), and *engrailed* (*en*), which are expressed

with a single-segment periodicity (Dinardo and O'Farell, 1987; Ingham et al., 1988; Bopp et al., 1989). In *prd* mutant embryos, the expression of Gsb, Wg, and En is abolished in even-numbered parasegments, which results in the loss of the posterior part of even-numbered parasegments and of the adjacent anterior part of the odd-numbered parasegments (Nüsslein-Volhard and Wieschaus, 1980; Bopp et al., 1989; Ingham and Martinez Arias, 1992).

In addition to its embryonic segmentation function, Prd is required for the survival of the embryo to adulthood and for male fertility (Xue and Noll, 1996; Xue et al, 2001; Xue and Noll, 2002). Two homologs of Prd, the *Drosophila* Gsb and mouse Pax3 proteins, share a highly conserved N-terminal half with Prd that includes the two DNA binding domains, but differ in their C-terminal portions. These homologs are able to perform some of the Prd functions when they are placed under the control of the complete *prd cis*-regulatory region (Xue and Noll, 1996; Xue et al., 2001). These results suggest that change(s) in enhancers rather than in the coding region were the major evolutionary force driving the functional diversification of these three genes (Xue and Noll, 1996). However, both evolutionary alleles of *prd*, *prd*-Gsb and *prd*-Pax3, perform the cuticle function of Prd at low efficiency, and only *prd*-Gsb is able to rescue the *prd* mutant embryos to adulthood when it is present in two copies (Xue and Noll, 1996). These observations indicate that the coding region also plays an important role in further adaptation of protein functions.

**Role of *prd* in development and function of accessory glands**

The *Drosophila* accessory gland, a functional homolog of the human prostate, is a secretory organ of the male reproductive system that plays an essential role in reproduction. It is responsible for the production and secretion of most of the seminal fluid components that include several enzymes, small peptides, glycoproteins, and lipids. The accessory gland secretions are crucial for the reproductive success of males and are transferred, together with the sperm produced by the testes, to females during copulation (Chen, 1984; Xue and Noll, 2000; Xue and Noll, 2002). In mated females, accessory gland proteins (Acps) enter the hemolymph and affect the nervous and/or endocrine systems that induce a number of physiological and behavioral responses. These include increased egg deposition, decreased sexual receptivity, improved sperm storage, increased appetite, diminished attractiveness to males and shortened life span (Chen, 1984; Chen, 1996; Wolfner, 1997; Wolfner, 2002). It was shown that a female's egg-laying upon mating is modulated by at least two different Acps: Acp26Aa (Ovulin) and Acp70A (Sex peptide). Ovulin stimulates the release of mature

oocytes from the ovary of mated females. Increased egg-laying and decreased sexual receptivity are mating-induced responses that last for about a week in *Drosophila melanogaster* and require sex peptide (Liu and Kubli, 2003). In addition, components of the seminal fluid are absolutely required for sperm fertility (Xue and Noll, 2000) and essential for the storage of sperm in the spermatheca of the female genital tract (Tram and Wolfner, 1999). It has been shown that at least two components of the male's seminal fluid, the glycoprotein Acp36DE and the protease inhibitor Acp62F are required for normal sperm storage in *Drosophila melanogaster* (Bertram et al., 1996; Mueller et al., 2008).

The accessory glands are a pair of dead-end tubes that join the anterior end of the ejaculatory duct and develop from a special set of cells in the male genital disc (Nöthiger et al., 1977). The developmental fate of these cells is determined by the male sex determination pathway during the third larval instar (Chapman and Wolfner, 1988). The accessory glands are composed of an inner layer of secretory cells that produces and secretes many components of the seminal fluid in to the lumen, and an outer sheath of muscle cells that squeeze the accessory gland and force the accessory fluid into the ejaculatory duct during mating. There are two morphologically distinct types of secretory cells, the 'main cells', which comprise 95% of the secretory cells, and the 40-50 'secondary cells' comprising the remaining 5% of the secretory cells (Bairati, 1968; Bertram et al., 1992). The main cells are flat, hexagonal, binucleate cells that surround the lumen of the glands. The secondary cells are large, spherical, binucleate cells with large vacuoles and interspersed between the main cells at the distal end of each lobe. Each cell type in the secretory layer mediates a subset of the post-mating responses in females by producing a characteristic set of Acps. Although the functions of the accessory gland fluid are well understood, little is known about the molecular mechanisms that specify accessory gland development and the regulation of its components. In addition to its embryonic segmentation function (Nüsslein-Volhard and Wieschaus, 1980), *prd* is necessary for post-embryonic viability and male fertility (Bertuccioli et al., 1996; Xue and Noll, 1996; Xue and Noll, 2000; Xue and Noll, 2002). Previous work on this male fertility function has shown that *prd* is crucial for the development of accessory glands (Bertuccioli et al., 1996; Xue and Noll, 2000). Prd plays a dual role in accessory gland development, an early function required for cell proliferation and the formation of accessory glands, and a late function controlling the expression of various accessory gland products including the Sex-peptide (Xue and Noll, 2002). The early function requires a motif present in the C-terminal half of Prd, whereas the late function depends on the DNA-binding

specificity of the highly conserved N-terminal region of Prd that cannot be replaced by the N-terminal moiety of Gsb (Xue et al., 2001). The enhancer necessary for both of these functions is located in the downstream *cis*-regulatory region of *prd* (Xue and Noll, 2002).

**Development of abdominal segments in the adult *Drosophila***

Each epidermal segment in *Drosophila* is subdivided into anterior and posterior compartments that are defined by the expression of the selector gene *en* (reviewed by Lawrence, 1992; Lawrence and Struhl, 1996). Cell interactions at the compartment boundary are crucial for pattern formation within the segment (Lawrence and Struhl, 1996; Tabata et al., 1995). Cells at the compartment boundary are associated with long-range organizing properties and thus act as signaling centers controlling the development of a major portion of the segment (Struhl et al., 1997). Under control of the *en* gene, cells in the posterior compartment secrete a short-range morphogen, Hedgehog (Hh). The Hh signal cannot move very far from its source of production, but crosses the compartment boundary and, following its interaction with the receptor protein Patched (Ptc) in the adjacent anterior compartment cells, activates long-range morphogens, the products of *decapentaplegic* (*dpp*) or *wg*. These long-range morphogens induced by *hh* control the patterning of both anterior and posterior compartments (Dougan and DiNardo, 1992; Lawrence et al., 1996; de Celis et al., 1996; Lecuit et al., 1996; Nellen et al., 1996).

Unlike the larval cuticle that is secreted by large, polyploid cells that derive from the epidermis of the mature embryo without cell division, the cuticle of the adult abdomen develops from a specialized set of cells, the so-called histoblasts, that are continuous with the larval epidermis (Madhavan and Madhavan, 1980). The histoblast nests are laid down during embryogenesis as derivatives of the embryonic epidermis (Simcox et al., 1991). There are three major histoblast nests in each larval abdominal hemisegment (Madhavan and Madhavan, 1980). Each histoblast nest undergoes many cell divisions and forms a specialized structure of each abdominal segment. The sternite and pleural cuticle develop from the ventral hostoblast nest, the anterior dorsal nest produces the tergite, while the intertergal cuticle develops from the posterior dorsal histoblast nest (Madhavan and Madhavan, 1980). Unlike cells of imaginal discs, abdominal histoblasts do not divide during larval stages but are arrested in the G2 phase of the cell cycle and increase in volume about 60-fold (Garcia-Bellido and Merriam, 1971). Upon pupariation, histoblasts undergo rapid cell division that allows them to expand and fuse laterally at the expense of the preexisting

surrounding polyploid larval epithelial cells that undergo apoptosis (Madhavan and Madhavan, 1980). Ecdysone signaling is required for both the proliferation of histoblasts and the cell death of larval epithelial cells (Ninov et al., 2007). Despite extensive studies on pattern formation in the embryo and wing disc, little is known about the pattern formation in the adult abdomen. Many segment-polarity genes, including *en*, *hh*, *wg*, and *patched* (*ptc*), are involved in patterning of the adult abdominal segments (Struhl et al., 1997; Lawrence et al., 1999). The expression pattern(s) of these genes in the larval epithelial cells (Struhl et al., 1997) is largely unchanged from those in the mature embryo (Hooper and Scott, 1992). The proneural genes of the *acheate-scute* complex function in histoblast cells in the same manner as in other epidermal tissues to control the bristle pattern in the abdominal segments of the adult fly (reviewed by Campuzano and Modolell, 1992). The same genes that establish the planar polarity in the eye and wing also control the orientation of bristles in the adult abdomen (Casal et al., 2006).

### *Cis*-regulatory elements of *prd*

A transgene consisting of an 18 kb genomic fragment (*prd*-SN20), including the transcribed portion, 10 kb upstream region and 5.7 kb downstream sequences of the *prd* gene, is able to direct *prd* expression in the embryo like the wild-type *prd* gene (Gutjahr et al., 1994). One copy of the *prd*-SN20 transgene rescues all *prd* mutants to viable adults and thus includes all crucial *cis*-regulatory elements of *prd* (Gutjahr et al., 1994). Extensive analysis of the *prd* upstream region revealed that there are multiple *cis*-regulatory elements that direct the stripe expression pattern in the early embryo (Gutjahr et al., 1994). The 10-kb upstream region of *prd* contains both activator and repressor binding sites that mediate the establishment of the pair-rule pattern as well as the splitting of each pair-rule stripe into anterior and posterior stripes to generate the 14-stripe segment-polarity-like expression pattern (Gutjahr et al., 1994). The *prd* intron in combination with a minimal upstream region (150 bp) is able to direct reporter gene expression in stripes. The upstream sequences alone are not sufficient to drive reporter expression in the dorsal spot (Gutjahr et al., 1994). Therefore, the downstream sequences of *prd* are expected to contain enhancers for the dorsal spot. The expression of *prd* is subject to a regulatory hierarchy among pair-rule genes and is regulated directly or indirectly by all pair-rule gene products (Baumgartner et al., 1990). Thus, the *prd* gene is at the bottom of this pair-rule gene hierarchy and mediates the transition from pair-rule to segment-polarity genes. The expression of *prd* is subject to a combinatorial regulation and

probably involves the cooperative binding of pair-rule and gap gene products to its *cis*-regulatory elements. Analysis of *prd* expression in gap mutants suggests that the products of the gap genes activate *prd* expression in stripes, which is subsequently refined by the products of pair-rule genes (Gutjahr et al., 1993). *prd* mutants rescued by a *prd* transgene, *prd*Res, that includes all upstream but presumably no downstream regulatory sequences are sterile and lack accessory glands (Bertuccioli et al., 1996; Xue and Noll, 2002). The downstream sequences of *prd* rescue the development of accessory glands as well as the male fertility function of *prd*. The enhancer necessary for this dual function has been mapped to a 500 bp fragment of the downstream region (Xue and Noll, 2002).

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# Chapter 2

# Analysis of *prd* enhancers


## Summary

The *Drosophila prd* gene, the founding member of the Pax gene family and member of the *Pax*-3/7 subfamily, is required for proper segmentation of the larval cuticle, survival to adulthood, and male fertility. Previous studies, using reporter transgenes of *prd*, showed a complex organization of embryonic segmentation enhancers in the upstream region of *prd* and an enhancer regulating the male fertility function in the downstream region of *prd*. Here, the *cis*-regulatory elements of *prd* were further characterized by the use of a series of reporter and *prd* transgenes under the control of an incomplete set of its enhancers that led to the discovery of several novel functions and expression patterns of *prd*. Our results demonstrate that, in addition to upstream enhancers, downstream and intronic enhancers of *prd* produce a striped pattern in the embryo. Investigation of these enhancers revealed a complex interplay between the upstream and downstream enhancers of *prd*. The downstream enhancers are partially redundant for cuticular and survival functions of *prd*. One copy of a *prd* transgene under control of the upstream enhancers is sufficient to rescue the cuticular function but haplo-insufficient for rescue of the survival function of *prd*. In addition, another accessory gland enhancer was discovered in the upstream region that is crucial for the male fertility function of *prd*. Finally, we could identify three enhancers responsible for *prd* expression in the dorsal spot of embryos, in the ventral organs of larvae, and in the trochanter of pupal and adult legs.

## Introduction

Position along the antero-posterior axis of the developing *Drosophila* embryo is initially defined by the sequential activities of four classes of segmentation genes – the maternal coordinate genes and the zygotic gap, pair-rule, and segment polarity genes – and the homeotic genes, which form a hierarchical network (Nüsslein-Volhard and Wieschaus, 1980; Lehmann and Nüsslein-Volhard, 1986; Baumgartner and Noll, 1990; Small et al., 1991; Bejsovec and Martinez Arias, 1991; St Johnston and Nüsslein-Volhard, 1992; Kornberg and Tabata, 1993).

The *paired* (*prd*) gene, initially identified as a pair-rule segmentation gene (Nüsslein-Volhard and Wieschaus, 1980), encodes a transcription factor containing two DNA binding domains, a paired-domain (PD) and an extended *prd*-type homeodomain (HD), in its N-terminal half (Bopp et al., 1986) and a His-Pro (PRD) repeat near its C-terminal end (Frigerio et al., 1986). Prd protein is initially expressed in a broad anterior stripe at the end of the thirteenth nuclear division of syncytial blastoderm (Gutjahr et al., 1993). By mid cellularization, Prd appears in an anterior dorsal patch and in a characteristic pair-rule pattern of seven stripes, which by cellular blastoderm are converted into 14 stripes spanning each parasegment boundary (Gutjahr et al., 1993). During germ band extension, Prd expression decreases in the epidermal stripes but later accumulates in a few specific cells of the central nervous system and certain head regions (Gutjahr et al., 1993). Together with other pair-rule genes, *prd* specifies position along each double-segment repeat and activates the segment-polarity genes, including *gooseberry* (*gsb*), *wingless* (*wg*), and *engrailed* (*en*), which are expressed at a single-segment periodicity (Gutjahr et al., 1993; Xue et al., 2001). In *prd* mutant embryos, every other stripe of Gsb, Wg, and En protein is abolished, which results in the loss of the posterior part of even-numbered parasegments and of the adjacent anterior part of odd-numbered parasegments (Gutjahr et al, 1993; Xue et al., 2001). Prd is required in vivo not only for the expression of segment-polarity genes and normal development of the larval cuticle but also for the survival of the embryo to adulthood and for male fertility (Xue and Noll, 1996; Xue and Noll, 2000; Xue and Noll, 2002).

Two Prd homologs, the *Drosophila* Gsb and murine Pax3 proteins, which share with Prd a highly conserved N-terminal moiety including the PD and HD, but have

divergent C-terminal portions, are able to perform many of these functions of Prd when placed under the control of the entire *prd cis*-regulatory region (Xue and Noll, 1996). It follows that the acquisition of new *cis*-regulatory elements rather than changes in the coding region is the major evolutionary drive for the functional diversification among these three genes. However, both *prd*-Gsb and *prd*-Pax3 perform the cuticle function of Prd at low efficiency, and only *prd*-Gsb is able to rescue the *prd* mutant embryos to adulthood when it is present in two copies (Xue and Noll, 1996). These results indicate that the coding region also plays an important role in further modification of protein functions.

In vitro experiments suggest that these two domains can function either independently or cooperatively when present in the same molecule (Treisman et al., 1991; Underhill et al., 1995; Jun and Desplan, 1996). In vivo results demonstrate that the PD and HD of Prd have to be present in *cis* for the activation of segment-polarity genes, wild-type cuticle formation, and viability (Xue et al., 2001). In addition to the PRD repeat, which constitutes an important activation domain facilitating all functions of Prd, its C-terminal moiety contains at least two essential functional motifs (Xue et al., 2001). One motif, required for its function in larval cuticle development, is present in the C-terminal portions of both Gsb and Pax3, whereas another motif, needed for its role in viability, is present only in the Gsb C-terminal half (Xue et al., 2001). Finally, the determinant for the male fertility function, which cannot be replaced by the *prd*-Gsb and *prd*-Pax3 transgenes, resides in the conserved N-terminal rather than the divergent C-terminal moiety of Prd (Xue et al 2001; Xue and Noll, 2002). This observation challenges the general view using the fraction of amino acid identities as the sole measure of functional equivalence between homologous proteins.

Rescue experiments of *prd* null mutants revealed that a rescue transgene of *prd, prd*-SN20, extending from 10-kb upstream to 5.7 kb downstream of the transcribed region of *prd*, rescues all *prd* functions in a single copy with a penetrance of more than 95% (Table 1; Gutjahr et al., 1993). The *prd*-SN20 transgene is expressed in the same pattern as the endogenous *prd* gene and thus comprises the entire *cis*-regulatory region of *prd* (Gutjahr et al., 1994). To analyze the *prd* upstream regulatory region in detail, a series of 5' deletions of *prd-lacZ* fusion genes was constructed whose expression was studied in vivo (Gutjahr et al., 1994). The results indicated that multiple elements in the *prd* 5'-flanking region are required to direct *lacZ* expression in stripes (Gutjahr et al., 1994). Here, we investigated the *cis*-

regulatory elements of *prd* in great detail, using a series of reporter transgenes and partial rescue transgenes. We could show that the downstream and intronic sequences of *prd* also produce a striped pattern in the embryo. The downstream enhancers are partially redundant for cuticular and survival functions of *prd*. One copy of a *prd* transgene under the control of all upstream sequences is sufficient to rescue the cuticular function of *prd* but haplo-insufficient for the survival function of *prd*. An accessory gland enhancer in the upstream region has been shown to be crucial for the male fertility function of *prd*. Finally, we could locate the enhancers responsible for *prd* expression in the dorsal spot in the embryos, ventral organ of the larvae, and the trochanter segment of the legs.

## Results

### The *prd*Res transgene is haplo-insufficient

The *prd* gene is expressed in a complex pattern during embryogenesis (Gutjahr et al., 1993). The full rescue construct of *prd*, *prd*-SN20, includes, in addition to its transcribed region, 9.7-kb upstream and 5.7-kb downstream sequences (Fig. 1; Gutjahr et al., 1993). The *prd*-SN20 transgene is expressed in the same pattern as the endogenous *prd* gene and thus comprises the entire *cis*-regulatory region of *prd* (Gutjahr et al., 1993). Rescue experiments using *prd* null mutants showed that *prd*-SN20 rescues all *prd* functions in a single copy with a penetrance of more than 95% (Table 1; Gutjahr et al., 1993). To analyze the *prd* upstream regulatory region in detail, a series of 5' deletions of *prd-lac*Z fusion genes was constructed whose expression was studied in vivo. The results indicate that multiple elements in the *prd* 5'-flanking region are required to direct fusion gene expression in stripes (Gutjahr et al., 1994).

To investigate the functional significance of *prd cis*-regulatory elements in great detail, we first used two incomplete *prd* transgenes: (i) *prd*Res, which includes the entire upstream region but only 0.5 kb of the downstream region (Fig. 1; Bertuccioli et al., 1996). (ii) *prd*-mf5, which includes the entire transcribed and 5.7-kb downstream regions of *prd*, but only 234 bp of the upstream region (Fig. 1; Xue and Noll, 2002). The *prd*Res transgene, which lacks almost the entire downstream region, rescues all *prd* null mutants to adulthood in two copies (Table 1), but males are sterile because they have no accessory glands (Bertuccioli

et al., 1996). The enhancer for accessory gland development has been mapped to the most distal downstream region of *prd* (Xue and Noll, 2002).

The *prd* mutant alleles used in these and all subsequent rescue experiments, unless otherwise mentioned, were a transheterozygous combination of two *prd* null alleles, $prd^{2.45}$ and $prd^{32.12}$ (Frigerio et al., 1986; Kilchherr et al., 1986; Bertuccioli et al., 1996). A quantitative analysis of rescue results obtained with *prd* transgenes showed that the *prd*Res transgene is haplo-insufficient (Table 1). One copy of *prd*Res rescues only 20-25% of *prd* mutants to adulthood (Table I) and exhibits a predominant male-specific rescue. About 90% of the *prd* mutants rescued by one copy of the *prd*Res transgene are males, which suggests that certain female functions of *prd* depend on high levels of Prd protein. Two copies of *prd*Res are sufficient to rescue more than 95% of *prd* mutants to viable adults (Table 1). One copy each of *prd*Res and *prd*-mf5, rescue more than 95% of *prd* mutants to viable adults as well (Table 1). These results demonstrate that the downstream enhancers are crucial for efficient survival of *prd* mutants. One copy of *prd*Res could rescue 50% of *prd* mutants to adulthood, which consisted, however, of equal numbers of males and females, when a hypomorphic allele, $prd^{IIN27}$, was combined with the null allele, $prd^{2.45}$ (Table 1). However, one copy of *prd*Res is sufficient to rescue all mutants of a weak *prd* hypomorphic allele, $prd^{IIB42}$, missing the 126 C-terminal amino acids of Prd, when used in combination with $prd^{2.45}$ (Table 1). In both cases, all rescued males are sterile and display underdeveloped accessory glands (data not shown).

**Minimization of *prd* enhancers in *prd*Res transgene**

The *prd*-SN20 and *prd*Res rescue constructs include part of the neighboring upstream gene, CG5325 (Fig. 1). It is possible that some *prd* enhancers also act on the promoter of CG5325 in the *prd*Res transgene and hence activate the *prd* promoter only weakly, which causes the haplo-insufficiency of the *prd*Res transgene. The sequences of the neighboring gene might further include *prd* enhancers necessary for some of the *prd* functions. To test these possibilities, we tried to minimize the upstream sequences of *prd*Res in *prd* transgenes that were tested for their rescue capabilities (Fig. 1). These new *prd* transgenes might also shed light on possible redundancies among *prd* enhancers. Rescue experiments with *prd*-SN20 and *prd*Res suggested that a *prd* transgene, *prd*Res1, including 9.7 kb of the upstream region, present in *prd*-SN20, and 0.5 kb of the downstream region of *prd*, present in *prd*Res, will behave like *prd*Res (Fig. 1). In *prd*Res2, upstream sequences that belong to the neighboring

gene were removed from the rescue construct, but the 0.5 kb downstream sequences were retained, while *prd*Res3 is derived from *prd*Res1 by deletion of the 0.5 kb downstream region (Fig. 1). In *prd*Res4, the 0.5 kb downstream sequences were removed from *prd*Res2 (Fig. 1). Rescue with the *prd*Res3 and *prd*Res4 transgenes tests whether the 0.5 kb downstream region contributes to the rescue efficiency of the *prd*Res transgene. The new *prd*Res transgenes were analyzed for their efficiencies to rescue *prd* null mutants to adulthood. Interestingly, all the new *prd*Res transgenes behaved like *prd*Res in rescue experiments, which demonstrates that the 0.5 kb downstream region and the part of the neighboring gene sequences do not contribute to functions present in *prd*Res (Table 1).

**Analysis of *prd*Res expression patterns in the embryos**

To test whether the haplo-insufficiency of *prd*Res occurs during embryonic development, we investigated the expression patterns of *prd*Res in *prd* null mutants. The early expression of *prd*Res in stripe 1 and the temporal appearance of *prd* stripes in these embryos is very similar to that in wild-type embryos (cf. Fig. 2I, J with Fig. 2A, B). However, the pair-rule and segment polarity patterns of *prd*Res (Fig. 2K-N) deviate significantly from the wild-type patterns of *prd* (Fig. 2C-F). The pair-rule stripes of *prd*Res are weaker (Fig. 2K) than those of *prd* (Fig. 2C). Prd expression in posterior stripes appears delayed but normal, whereas the anterior stripes remain very weak (cf. Fig. 2K-N with Fig. 2C-F). The anterior dorsal spot is not expressed by *prd*Res (cf. Fig. 2K-O with Fig. 2C-G). These results suggest that the enhancer(s) controlling *prd* expression in the dorsal spot are located in the downstream region of *prd*, in agreement with an earlier analysis from this lab (Gutjahr et al., 1994). However, two copies of *prd*Res rescues all *prd* null mutants to adulthood (Table 1), which demonstrates that dorsal spot expression is not crucial for survival. Late expression of *prd*Res in the head region of embryos (Fig. 2P) is again similar to wild-type expression of *prd* (Fig. 2H). The weak anterior stripes of *prd*Res suggest that the downstream region of *prd* contains enhancers that activate *prd* expression in the anterior stripes.

**One copy of *prd*Res is sufficient to rescue the cuticular phenotype of *prd* mutants**

The analysis of *prd*Res expression patterns in embryos showed weak *prd* anterior stripes. However, previous studies suggested that all known targets of *prd* overlap with the posterior but not with the anterior stripes (Xue et al., 2001). Therefore, it seemed unlikely that the anterior stripes contribute to the cuticular function of *prd*. To further test whether the weak anterior stripes are responsible for the haplo-insufficiency of the *prd*Res transgene, we

analyzed the cuticular phenotype of *prd* mutants rescued by only one copy of *prd*Res. Embryos that lack *prd* function exhibit the classical pair-rule phenotype in their cuticle (Fig. 3B; Nüsslein-Volhard and Wieschaus, 1980), in which segmental equivalents are deleted with a double-segment periodicity as compared to wild-type embryos (Fig. 3A). This phenotype results from the failure to express several segment-polarity genes, like *gsb, wg*, and *en*, on either side of every other parasegmental boundary (Ingham and Martinez Arias, 1992). Surprisingly, one copy of *prd*Res is sufficient to rescue the cuticular phenotype of *prd* mutant embryos completely (Fig. 3C). These results suggest that the weak anterior stripes in *prd*Res embryos are dispensable for the cuticular function of *prd*.

***prd* is required for post-embryonic viability and survival to adulthood**

Previous work from our lab has shown that two evolutionary alleles of *prd*, *prd*-Gsb and *prd*-Pax3, which share with *prd* the same *cis*-regulatory region but not identical coding sequences, have conserved most of the functions of *prd*. While both *prd*-Gsb and *prd*-Pax3 can support normal cuticle development, only *prd*-Gsb is able to rescue about 10% of *prd* mutants to adulthood when present in two copies (Xue and Noll, 1996; Xue et al., 2001). These results suggest that *prd* has a function required for post-embryonic viability, which is not fully supported by its evolutionary alleles. The motif required for cuticle formation is retained in both Gsb and Pax3, the viability function only in Gsb.

Rescue experiments with *prd*Res demonstrate that one copy of *prd*Res is not sufficient to rescue the post-embryonic viability function of *prd* completely (Table 1). To investigate this haplo-insufficiency, the hatching frequency of *prd* mutants rescued with one copy of *prd*Res was carefully measured. The analysis showed that *prd* mutant larvae rescued by one copy of *prd*Res hatch with an efficiency comparable to that of wild type. We then investigated larval development of *prd* mutants, rescued by one copy *prd*Res, whereby a $y^+$ marked *CyO* balancer chromosome was used in a *y w* genetic background to isolate transheterozygous *prd* mutant larvae rescued by one copy of *prd*Res. All $y^-$ (*y w*; $prd^{2.45}/prd^{32.12}$; *prd*Res/+) larvae were separated during the first instar and transferred to a tube with fresh fly food. The development of these larvae was monitored over the time. About 70% of the *prd* mutant larvae rescued by one copy of *prd*Res show a strong developmental delay, an extended third instar stage, and a small size (Fig. 3D). These small larvae die during late third instar. The remaining 30% *prd* mutants rescued with one copy of *prd*Res develop normally and reach adulthood (Fig. 3D).

**Analysis of downstream enhancers of *prd***

Based on previous studies from this lab, the downstream region of *prd* is expected to contain enhancers for stripe 1 and dorsal spot expression (Gutjahr et al., 1994). To analyze the expression pattern activated by the downstream enhancers, we initially used the *prd*-mf5 transgene. However, the expression pattern of *prd*-mf5 does not reflect the precise expression pattern activated only by downstream enhancers, since the 234 bp upstream region and the *prd* intron of *prd*-mf5 are sufficient to generate weak *prd* stripes (Gutjahr et al., 1994). Therefore, a *prd*-lacZ reporter construct was prepared that included the entire downstream region but is expressed under the control of the P-element promoter (Fig. 4). In contrast to the *hsp70* basal promoter, this promoter is, according to our experience, promiscuous or permissive in the sense that it interacts with specific enhancers of any gene to activate transcription of the gene that is faithful in space and time, i.e., indistinguishable from that obtained under control of its cognate promoter. Consistent with our earlier hypothesis (Gutjahr et al., 1994), LacZ is expressed in the dorsal spot (Fig. 4H-K) and weakly in stripe 1 (Fig. 4G). In addition, we observed the characteristic yet weak early 14-stripe pattern (Fig. 4H, I), in which every other stripe was enhanced at later stages (Fig. 4J, K) and which resembles the late expression of *prd* in these stripes (Fig. 4F). This 14-stripe pattern and the expression in the dorsal spot and stripe 1 are similar to the Gal4/UAS-GFP expression pattern of *prd*-mf5 in a $prd^{+}$ background (Fig. 6A, E). The 14-stripe Gal4/UAS-GFP expression pattern of *prd*-mf5 is quite patchy (Fig. 6E), probably because the Gal4 expression in *prd*-mf5 is driven by an *hsp70* basal promoter (Fig. 1). It remains to be tested whether in a $prd^{-}$ background the 14-stripe pattern of *prd* 3.0-*lacZ* changes to a 7-stripe pair-rule pattern, as observed for Prd expression of *prd*-mf5 in $prd^{-}$ embryos, the only genetic background in which it can be analyzed (data not shown). However, if this 7-stripe pair-rule pattern of *prd*-mf5 in $prd^{-}$ embryos is generated entirely by the intronic enhancer (Fig. 7D-I), then it is likely that the 14-stripe pattern of *prd* 3.0-*lacZ* disappears completely in a $prd^{-}$ background. In such a case, the expression level of the 14-stripe pattern of *prd* seems to depend on autoregulation through the downstream enhancers, which are missing in the *prd*Res transgene used to rescue $prd^{-}$ embryos, in which the anterior stripes are clearly weaker and even the posterior stripes might be slightly affected (Fig 2). However, the expression pattern of *prd*-mf5 in the dorsal spot and stripe 1 appears to be unaffected by the $prd^{-}$ background (data not shown).

**Mapping of enhancers for dorsal spot and striped expression in the downstream region**

We have shown that *prd* functions regulated by downstream enhancers are crucial for survival in the presence of only one copy of a *prd* transgene regulated only by *prd* upstream sequences, but dispensable for survival in the presence of two copies of such a *prd* transgene (Table 1). In order to understand the function(s) of downstream enhancers in detail, we have mapped the enhancers in the downstream region by a series of *prd*-mf transgenes (Fig. 5; Xue and Noll, 2002). All *prd*-mf transgenes include a *prd* promoter of 234 bp upstream of the transcriptional start site and the *prd* intron, which are sufficient to produce a weak stripe pattern (Fig. 5; Gutjahr et al., 1994; Xue and Noll, 2002). Therefore, the expression patterns of *prd* produced by these *prd*-mf transgenes could not be used for this purpose. However, the expression of Gal4 of these transgenes could be used to map the enhancers, as Gal4 expression is regulated by the same downstream sequences as *prd* but under control of the *hsp70* basal promoter (Fig. 5). The *prd*-mf transgenic flies were crossed with UAS-GFP flies and GFP expression was analyzed during embryonic stages. The expression patterns revealed that *prd*-mf5 and *prd*-mf4, but not *prd*-mf3 (not shown), show stripe 1 expression (Fig. 6A-B). These results map an essential portion of the stripe 1 enhancer to the *Bam*HI-*Eco*RI fragment of the downstream region (Fig. 5). The 14-stripe expression pattern of *prd*-mf5 is retained completely in *prd*-mf4 but only partially in *prd*-mf3 (Fig. 6C, E, F). The *prd*-mf2 transgene exhibits strong ectopic expression in the head region but no clear stripe patterns in the abdomen (Fig. 6G). Based on these results, the enhancer necessary for *prd* expression in stripes is mapped to a downstream region located between *Pst*I and *Eco*RI (Fig. 5). The dorsal spot expression was seen in all *prd*-mf transgenes analyzed (Fig. 6C-G). Therefore, the enhancer controlling *prd* expression in the dorsal spot resides between *Xba*I and *Xho*I (Fig. 5).

**Mapping of survival enhancer in the downstream region of *prd***

The results of this study show that the *prd*Res transgene is haplo-insufficient. One copy of *prd*Res rescues only 20-25% of *prd* mutants to adulthood, while two copies of *prd*Res are sufficient to rescue more than 90% of *prd* mutants to viable adults (Table 1). One copy each of *prd*Res and *prd*-mf5 rescue more than 95% of *prd* mutants to adulthood (Table 1). To find out which part of the *prd*-mf5 transgene contributes to the survival of *prd* mutants, we examined the survival of *prd* mutants rescued by one copy each of *prd*Res and a *prd* transgene under the control of various partial downstream regions of *prd* (Fig. 5; Xue and Noll, 2002). These transgenes rescue *prd* functions mediated by the downstream enhancers

and serve as Gal4 reporters because they drive the expression of Prd as well as Gal4 proteins under the control of the same *cis*-regulatory regions. As shown above, the *prd*-mf5 transgene, together with one copy of *prd*Res, rescues all *prd* mutants to adulthood. This function is retained fully in *prd*-mf4, *prd*-mf3, and *prd*-mf2 but only partially in *prd*-mf1 (Fig. 5). The *prd*-mf1 transgene, together with one copy *prd*Res, rescued only 50% of *prd* mutants (Fig. 5). However, the males rescued with *prd*-mf4, *prd*-mf3, *prd*-mf2, and *prd*-mf1 are all sterile, as they are lacking the accessory glands. Based on these results, we mapped the survival enhancer to a region between *Xba*I and *Pst*I of the *prd* downstream region (Fig. 5). Thus, it is located proximal to the stripes enhancer of the downstream region.

These results suggest that the striped pattern regulated by downstream sequences does not enhance the survival of *prd* mutants in the presence of only one copy of *prd*Res and are consistent with the fact that one copy of *prd*Res is sufficient to rescue the cuticular phenotype of *prd* mutants completely (Fig. 3C). It is therefore likely that post-embryonic expression of *prd* controlled by downstream enhancers plays a major role in the survival of *prd* mutants.

**The *prd* intron contains enhancer sequences that produce a pair-rule stripe pattern**

A *prd*-lacZ reporter gene, under the control of 150 bp of the upstream promoter region and the intron of *prd*, exhibits a weak pair-rule stripe pattern during late syncytial blastoderm and germ band extension, which suggests that the intron of *prd* includes a "*prd* zebra element" (Gutjahr et al., 1994). Therefore, a series of "minimal" *prd* transgenes with or without intron was tested, in which the upstream region was progressively reduced from 150 bp to 20 bp (Fig. 7A). Analysis of the expression patterns of these transgenes in $prd^{-}$ embryos showed that the shortest transgenes, including 20 bp or 35 bp of upstream sequences as well as the intron, produce similar pair-rule patterns of stripes as the transgene including 131 bp of the upstream region and the intron (Fig. 7B-H). However, this expression pattern is completely abolished in the absence of the intron (Fig. 7I). These results show that there are no enhancer sequences in this short upstream region and that the enhancer responsible for the pair-rule stripes is located in the intron of *prd*. In addition, the short upstream region and the adjacent 5' UTR must include a *prd* promoter sufficient to generate a weak pair-rule pattern of stripes when combined with the intron enhancer. This 7-stripe pattern produced by the intronic enhancer in a $prd^{-}$ background (Fig. 7B-H) is similar to the expression pattern of *prd*Δ*N* and *prd*Δ*Q* in a $prd^{+}$ background (Gutjahr et al., 1994). The *prd*Δ*N* and *prd*Δ*Q* are two *prd-lacZ* reporter transgenes, which contain 2 kb or 150 bp, respectively, of the upstream region and

the complete intron (Fig. 11A). These results indicate that the intronic enhancer is not subject to autoregulation.

The *prd* intron is included in the *prd*-SN20 transgene but not in *prd*-Gsb, a transgene in which the *prd* coding region in *prd*-SN20 is replaced by that of the *gsb* gene. Therefore, we wonder whether the presence or absence of the *prd* intron partly contributes to the observed differences in rescue efficiency between these two transgenes. However, the fact that a *prd*-Prd transgene that lacks only the intron is indistinguishable from *prd*-SN20, while *prd-int*-Gsb behaves the same as *prd*-Gsb clearly excludes such a possibility and demonstrates that the intron is dispensable for the *in vivo* functions of *prd* (Xue et al., 2001).

**Partial rescue of *prd* mutant cuticular phenotype by the *prd*-mf5 transgene**

Since enhancers in the *prd* intron or downstream sequences also support a striped expression pattern (Figs. 4 and 7), we were interested whether this expression pattern can rescue the cuticular phenotype of $prd^{-}$ embryos. The *prd*-mf5 transgene, which includes both downstream and intronic sequences was used to rescue the cuticular phenotype of $prd^{-}$ embryos. Analysis of cuticles revealed only a partial rescue: $prd^{-}$ embryos rescued by one copy of *prd*-mf5 showed very little or no rescue, with a minor fraction displaying partial or full rescue in two abdominal segments (Fig. 8B-D), whereas two copies of *prd*-mf5 displayed a considerable rescue of the cuticular phenotype except in the posterior abdominal region (Fig. 6F-H). The incomplete rescue in the posterior segments even in the presence of two copies of *prd*-mf5 transgene indicates the requirement of high Prd protein levels in this region. The most anterior region of the larval cuticle is not rescued either by the *prd*-mf5 transgene.

**Mapping of the enhancer for *prd* expression in the ventral organ**

The cuticular function of *prd* can be rescued by its two evolutionary alleles, *prd*-Gsb and *prd*-Pax3, when expressed under the complete *prd cis*-regulatory region (Xue and Noll, 1996). However, neither Gsb nor Pax3 are able to rescue the lethality of *prd* mutants completely (Xue and Noll, 1996). Two copies of *prd*-Gsb can rescue only 10% of *prd* mutants to adulthood (Xue and Noll, 1996). These results demonstrate that *prd* is required for post-embryonic viability, a function that cannot be provided with the same efficiency by Gsb or Pax3. To obtain some insight into the post-embryonic function(s) of *prd*, we investigated the post-embryonic expression patterns of *prd*. Analysis of *prd*-Gal4 expression patterns during

larval stages revealed that a *prd*-Gal4 transgene, in which Gal4 is expressed under the control of the *prd* promoter and the adjacent 9.7 kb upstream region, *prd5.1-Gal4* (Fig. 9A), showed expression in the ventral organ (Fig. 9B-D). Expression of *prd* in the ventral organ is corroborated by immunohistochemical staining for Prd with an anti-Prd antiserum (Fig. 9H-J).

The ventral organ consists of one chemosensory sensillum, innervated by four chemosensory neurons, and four mechanosensory sensilla, each innervated by one mechanosensory neuron. The chemosensory neurons of the ventral organ express *prd*, as indicated by their axonal projections (Fig. 9B). The sensilla of the ventral organ are characterized by a terminal pore, which indicates a gustatory function (reviewed by Stocker, 1994). Since investigation of the *prd* function in the developing ventral organ might uncover the post-embryonic function of *prd*, we have mapped the enhancer in the upstream region of *prd* that regulates expression in the ventral organ, using a series of *prd-Gal4::VP16* transgenes that are expressed under the control of different fragments of the upstream region (Fig. 9A). Investigation of the Gal4 expression patterns of these transgenes showed that a *prd*-Gal4 transgene containing 2.2 kb of the distal upstream region (between 7.7 kb and 5.5 kb from the transcriptional start site; fragment 1 in Fig. 9A) drives UAS-GFP expression in the ventral organ (Fig. 9E-G). To understand the function of *prd* in the development of the ventral organ, a partial rescue is planned with a *prd* transgene in which this sequence is deleted from the upstream region.

**Mapping of the enhancer for *prd* expression in legs**

Investigation of *prd*-Gal4/UAS-GFP expression patterns in adults revealed expression in the trochanter segment of the leg (Fig. 10B). The expression is detectable in all six trochanter segments, starting from mid-pupal stages (Georgijevic, Xue, and Noll, unpublished data). To understand the function of *prd* in leg development, the enhancer was mapped in the upstream region of *prd*, using the same series of *prd-Gal4::VP16* transgenes as above (Fig. 10A). Based on the analysis of UAS-GFP expression under the indirect control of these transgenes, the enhancer activating *prd* transcription in the trochanter segment of the leg was mapped to a 2.2 kb fragment between 5.7 kb and 3.5 kb in the upstream region of *prd* (Fig. 10A). A partial rescue is planned with a *prd* transgene in which this sequence is deleted from the upstream region to to investigate this function of *prd* in leg development.

**Analysis of an accessory gland enhancer in the upstream region of *prd***

The *prd* gene, in addition to its role in promoting segmentation, has been shown to be required for male fertility (Xue and Noll, 2002). Investigation of the male fertility function of *prd* revealed that it plays a dual role in accessory gland development and function (Xue and Noll, 2002). *prd* is required for accessory gland formation by promoting cell proliferation and is necessary for the expression of secretory gland products in adult accessory glands (Xue and Noll, 2002). Both these functions require an enhancer located in the downstream regulatory region of *prd* (Xue and Noll, 2002). However, it is not clear whether this male fertility function regulated by the enhancer in the downstream region is sufficient for the development and function of mature accessory glands, i.e., whether or not upstream enhancers contribute to this function of *prd*. The requirement of the upstream regulatory region of *prd* was never tested for the male fertility function of *prd* since this region is crucial for the survival of *prd* mutants to adulthood. The old observation that *prd* mutant males rescued by two copies of *prd*Res completely lack accessory glands demonstrates only that the upstream region is not sufficient, but not whether or not it is necessary, for the initiation of accessory gland development (Xue and Noll, 2002). Interestingly, the *prd*-mf5 transgene (Fig. 11A) drives UAS-GFP expression only at the tip of the gland, even in 1-day old virgin males (Fig. 11B, C), in contrast to the ubiquitous expression of Prd in the entire gland of wild-type males (Xue and Noll, 2002). This observation prompted us to investigate the expression patterns in accessory glands driven by the upstream region of *prd*. Gratifyingly, *prd*5.1-Gal4, a transgene regulated by the complete upstream region of *prd* (Fig. 11A), drives UAS-GFP expression ubiquitously in the accessory glands of 1-day old virgin males (Fig. 11E). Later this expression is restricted to few cells at the tip of the gland (Fig. 11F). Similarly, a *lacZ* reporter construct under control of the complete upstream region of *prd*, *prdDC* (Fig. 12A; Gutjahr et al., 1994), is expressed ubiquitously in accessory glands of 1-day old male virgins (Fig. 11D) but restricted in its expression to the tip of the glands in 5-day old virgin males (Fig. 11G). These results suggest that the upstream region of *prd* might be crucial for the male fertility function of mature accessory glands. The downstream region plays a major role in the development of accessory glands.

To further analyze the function of the *prd* upstream enhancer in male fertility, it was mapped by the use of a series of *lac*Z transgenes under control of decreasing lengths of the *prd* upstream region (Fig. 12A; Gutjahr et al., 1994). Based on the LacZ expression patterns,

the enhancer was mapped to a 660 bp fragment between *prdΔO* and *prdΔP* in the upstream region (Fig. 12B-E). To further test the requirement of this upstream region for the male fertility function of *prd*, it was deleted from the *prd*Res construct to generate the partial rescue construct, *prd*-mfdelup (Fig. 13A).

Since the male fertility enhancer in the downstream region is necessary for the development of accessory glands (Xue and Noll, 2002), the *prd*-mf5 transgene was used together with *prd*-mfdelup to rescue *prd* mutants. The results showed that deletion of the upstream enhancer that regulates transcription in adult accessory glands had no effect on the survival of *prd* mutants, but all rescued males were sterile. Analysis of these sterile males showed that their accessory glands were poorly developed and smaller (Fig. 13F, G) than those of wild-type males (Fig. 13B, C). The lumen appears normal in 1-day old rescued males (cf. Fig. 14B with Fig. 14A), but gradually disappears by 5 days (cf. Fig. 14C, E, F with Fig. 14D). This disappearance of the lumen might result from a failure of early *prd* target genes to be expressed, many of which secrete their products into the lumen. As a consequence, the integrity of the lumen is lost which in turn results in the collapse of the lumen. The accessory glands of 1-day old rescued virgin males showed a dramatic reduction of Prd expression (Fig. 13F, G) as compared to those of 1-day old wild-type virgin males (Fig. 13B, C). In contrast to 5-day old wild-type virgin males, however, the expression of Prd in 5-day old rescued virgin males is not restricted to the tip of the gland (compare Fig. 13H, I with Fig. 13D, E). Instead, an elevated level of *prd* expression was observed throughout the accessory gland of these males even after 2 weeks (data not shown). The *prd*-mfdelup transgene (Fig. 13A) does not exhibit such a persistent and strong expression of *prd* in a wild-type genetic background, which suggests that the deletion does not include silencer elements whose deletion might result in ectopic expression of the *prd* transgene (data not shown).

## Discussion

The detailed analysis of *prd* enhancers in this study revealed that there are at least three regions in the *prd* locus that can produce a stripe pattern independently of each other (Fig. 15). Previous studies from our lab have demonstrated that multiple *cis*-regulatory elements

in the upstream region produce a stripe pattern in the embryo. Here, we could show that the downstream region and intron sequences also produce striped patterns (Figs. 4 and 7). Investigation of *prd* enhancers in detail uncovered a partial redundancy between downstream, intronic, and upstream enhancers of *prd* (Figs. 2-4, 7, 8). In the presence of the upstream enhancers, downstream and intronic enhancers are redundant for proper development of the larval cuticle (Figs. 3C and 8) and partially redundant for survival to adulthood (Table 1 and Fig. 5). This partial redundancy plays an important role in the survival of *prd* mutants when only one copy of the *prd*Res transgene is present, which is under the sole control of the *prd* upstream region. In this study, we also isolated and mapped the enhancers controlling *prd* expression in the dorsal spot, ventral organ, and trochanter segment of the leg (Fig. 15). The enhancers controlling *prd* expression in stripes of the downstream region and intron of *prd* were mapped (Fig. 15). An additional enhancer in the upstream region has been shown to be required for the male fertility function of *prd* (Fig. 15).

In this study, we showed that the *prd*Res transgene is haplo-insufficient. Expression of *prd*Res in embryos showed weak anterior stripes, while the dorsal spot is completely absent (Fig. 2). However, the complete rescue in all embryos of the $prd^{-}$ cuticular phenotype by one copy of *prd*Res shows that the strong reduction of anterior stripe expression in *prd*Res embryos does not significantly affect the cuticular function (Fig. 3). The fact that the expression of none of the known *prd* target genes overlaps with the anterior stripes further questions their functional significance. We could also show that the dorsal spot expression is dispensable for the survival of *prd* mutants because two copies of *prd*Res are sufficient to rescue all *prd* nutants to adulthood (Table 1). All *prd* mutants rescued by one copy of *prd*Res hatch normally. The haplo-insufficieny of *prd*Res is explained by a post-embryonic function of *prd*, which cannot be fully rescued by one copy of the *prd*Res transgene. Interestingly, the downstream *cis*-regulatory region driving *prd* expression rescues the haplo-insuffiecieny of the *prd*Res transgene. The strong developmental delay and small size of *prd* mutant larvae rescued by one copy of *prd*Res (Fig. 3D) might result from a defect in feeding during larval stages. Further analysis revealed that *prd* is expressed in the ventral organ and in few supporting cells ventral to the mouth hooks during larval stages (Fig. 9). As the ventral organ has been proposed to be required for gustation in the larva (reviewed by Stocker, 1994), the expression of *prd* in the ventral organ might be important for the feeding behavior of the larva.

Two Prd homologs, the *Drosophila* Gsb and murine Pax-3 proteins, are able to perform some of the Prd functions when placed under the control of the entire *prd cis*-regulatory region. These results suggest that the acquisition of new *cis*-regulatory elements rather than changes in the coding region is the major evolutionary drive for the functional diversification of the genes encoding these homologous proteins (Xue and Noll, 1996). Both *prd*-Gsb and *prd*-Pax3 perform the cuticular function of Prd with low efficiency, when present as one copy, but exhibit full rescue when present as two copies. However, *prd*-Pax3 cannot rescue the *prd* mutants to adulthood, and only *prd*-Gsb is able to rescue 10% of *prd* mutants to adulthood when present as two copies (Xue and Noll, 1996). The fact that the *prd*-GsbN+PrdC transgene fully rescues *prd* mutants to adulthood suggests that this function requires the C-terminal portion of Prd (Xue et al., 2001). Unlike the cuticular function, the post-embryonic viability function of *prd* cannot be fully rescued by its evolutionary alleles, which demonstrates that the coding region also plays an important role in the adaption of a gene's function.

Rescue experiments indicated that the enhancers of *prd* are partially redundant for certain functions of *prd* (Table 1). Analysis of transgene expression patterns showed that not only the upstream enhancers but also the downstream and intronic enhancers produce a stripe pattern during embryonic stages. Preliminary evidence from the analysis of downstream enhancers indicated that *prd* regulates its own expression to generate the 14-stripe pattern. Since other pair-rule genes exhibit autoregulation (Harding et al., 1989; Jiang et al., 1991; Schier and Gehring, 1992), it will be interesting to investigate whether not only the downstream but also the complete upstream enhancer regulating transcription in stripes is subject to autoregulation. However some parts of the *prd* expression pattern are not affected by autoregulation, specifically the 7-stripe pattern regulated by the intronic enhancer (Fig. 7) and the *prd* expression in the dorsal spot and stripe 1 (data not shown). The enhancers controlling expression in the dorsal spot, stripe 1, and the 14-stripe pattern in the embryo were mapped to the downstream region in $prd^{+}$ embryos by taking the advantage of a *Gal4* transgene regulated by the same enhancers as *prd* in *prd*-mf transgenes (Fig. 5, Fig. 6, and Fig. 15). The cuticular rescue of $prd^{-}$ embryos by *prd*-mf5 suggests that the downstream and intronic enhancers can only partially substitute this function (Fig. 8). The presence of *prd*-mf5 in trans rescues the haplo-insufficiency of *prd*Res and enhances the survival of *prd* mutants to adulthood (Table 1). The enhancer necessary for this survival function has been mapped in the downstream region (Fig. 5). Interestingly, the survival enhancer in the

downstream region does not overlap with the 14-stripe enhancer (Figs. 7 and 15), which suggests that the survival function can be uncoupled from the cuticular function of *prd*.

With a series of *prd* transgenes with or without intron and including only a very short upstream region, we could show that the intron of *prd* harbors an enhancer, which can produce a 7-stripe pattern in embryos (Fig. 7). However, the enhancer in the intron is completely redundant and dispensable for in vivo functions of *prd*. One copy of the *prd*-Prd transgene, derived from *prd*-SN20 by deleting the intron, is sufficient to fully rescue all the *prd* functions, i.e., proper development of the larval cuticle, viability, and male fertility (Xue et al., 2001). In addition, the *prd-int*-Gsb transgene, in which the *prd* intron was inserted into *prd*-Gsb between the *prd* leader and *gsb* cDNA sequences, is functionally indistinguishable from *prd*-Gsb (Xue et al., 2001).

Rescue of *prd* mutants by *prd*Res, which lacks the distal 5.2 kb of the downstream region of *prd*-SN20, uncovered the male fertility function of *prd*. This function was not conserved in the evolutionary alleles, *prd*-Gsb and *prd*-Pax3, as *prd* mutant males rescued by *prd*-Gsb, or a combination of *prd*-Pax3 with *prd*-Gsb, are sterile and possess severely reduced accessory glands. Investigation of the male fertility function of *prd* revealed that *prd* plays a dual role in the development and function of accessory glands. The enhancer necessary for this male fertility function has been mapped to the distal part of the *prd* downstream region (Xue and Noll, 2002). However, it was not clear whether this enhancer is sufficient to control the male fertility function in the absence of any contribution from enhancers located in the upstream region of *prd*. Investigation of *prd*-Gal4 expression patterns indicated that the downstream region is not sufficient to recapitulate *prd* expression in adult accessory glands (Fig. 11). Indeed, we could show that the upstream region regulates *prd* expression in accessory glands (Fig. 11D-G). Further analysis demonstrated that the upstream enhancer is necessary for the male fertility function of *prd*. *prd* mutant males rescued by *prd*-mfdelup, a transgene devoid of this upstream enhancer, and a copy of *prd*-mf5 were sterile and displayed underdeveloped accessory glands (Fig. 13). The reduced expression of *prd* and the small size of these accessory glands suggest that the upstream enhancer is crucial for the late function of *prd* but not during the initial stages of accessory gland specification, which depends on the control by the downstream enhancer, as no accessory glands develop in its absence. The strong and persistent expression of *prd* in 5-day old virgin males indicates that the feedback regulation is not functional in these glands. The

initial failure to express some of the *prd* target genes at sufficiently high levels triggers the disintegration of the lumen in these flies that lack the control of *prd* by the upstream accessory gland enhancer (Fig. 14), which in turn might affect the feedback regulation.

## Materials and Methods

### Construction of transgenes

The initial cloning steps were carried out in the Bluescript (pKS$^+$) vector. Subsequently, cloned DNA fragments were recovered from the pKS$^+$ derivatives and cloned into P-element transformation vectors. The *prd* upstream region, including the promoter, and the leader were excised as *Not*I-*Hind*III fragment from the *prd*-SN20 construct (Gutjahr et al., 1994) and cloned into the pKS$^+$ vector to generate pKS$^+$-*prd*up. The *Hind*III-*Xba*I fragment, including part of the leader, the entire coding region, and the trailer of the *prd* gene, was excised from *prd*-SN20 and cloned into the pKS$^+$ vector to produce pKS$^+$-*prd*CDS. Subsequently, the *Not*I-*Hind*III and *Hind*III-*Xba*I fragments were excised from pKS$^+$-*prd*up and pKS$^+$-*prd*CDS, respectively, and ligated together into the *Not*I/*Xba*I-digested pW8 P-element transformation vector to obtain the pRes1 construct. For preparation of the pRes2 construct, first the *Eco*RI-*Hind*III fragment was excised from pKS$^+$-*prd*up and cloned into the *Eco*RI/*Hind*III-digested pKS$^+$ vector to produce pKS$^+$-*prd*up3.3. The pKS$^+$ plasmid containing the 7.7-kb *Eco*RI-*Hin*dIII fragment of the upstream and adjacent leader region of *prd,* pKS$^+$-*prd*7.7, was obtained by cloning the 4 kb *Eco*RI fragment from *prd*-*prd*up into the *Eco*RI site of the pKS$^+$-*prd*up3.3. The correct orientation of the *Eco*RI fragment in the pKS$^+$-*prd*7.7 construct was confirmed by sequencing. Finally, the *Eco*RI-*Hind*III fragment was excised as *Not*I-*Hind*III fragment from pKS$^+$-*prd*up7.7 and ligated together with the *Hind*III-*Xba*I fragment from pKS$^+$-*prd*CDS into a *Not*I, *Hind*III-digested pW8 vector to generate the pRes2 construct. To construct pRes3, the *Hind*III-*Bsi*WI fragment, including the coding region and the polyA addition site of *prd*, was first excised from pKS$^+$-*prd*CDS and subcloned into the pKS+ vector to generate pKS$^+$-*prd*CDSBsi. Subsequently, the *Not*I-*Hind*III fragment from pKS$^+$-*prd*up and *Hind*III-*Bsi*WI fragment, excised as *Hind*III-*Xba*I fragment from pKS$^+$-*prd*CDSBsi, were cloned together into a *Not*I, *Xba*I-digested pW8 vector to generate pRes3. For pRes4, the *Not*I-*Hind*III fragment from pKS$^+$-*prd*up7.7 and *Hind*III-*Bsi*WI fragments from pKS$^+$-prdCDSBsi were cloned together into a pW8 vector.

To obtain the *prd* 3.0-*lac*Z construct, first the *Bsi*WI-*Sal*I fragment, including the entire downstream region of *prd*, was excised from *prd*-SN20 and cloned into the pKS$^+$ vector. Subsequently, this fragment was excised as *Not*I-*Sal*I fragment and inserted between the *Not*I and *Xho*I sites, located downstream of the P-element promoter and *lac*Z coding region in the placW8 vector (a gift from Sabarinadh Chilaka).

To generate the the *prd* constructs that include various very short upstream regions and the intron of *prd*, the genomic *Pvu*II-*Bsi*WI fragment, extending from the *Pvu*II site in the upstream region to the *Bsi*WI site nearly at the end of the trailer of the *prd* gene, was first cloned from *prd*-SN20 into the pKS$^+$ vector to generate pKS$^+$-*prd*0. The individual fragments, consisting of decreasing lengths of the *prd* upstream region, were excised as *Bst*EII-*Bsi*WI, *Aat*II-*Bsi*WI, *Eco*R47III-*Bsi*WI fragments from pKS$^+$-*prd*0 and cloned each into the pW8 vector to generate the constructs, shown in Fig. 7, extending to -131, -95, and -35 bp of the upstream region.

To obtain the construct without intron, the *Hind*III-*Pst*I fragment of the c7340.6 *prd*-cDNA (Frigerio et al., 1986) was used to replace the corresponding fragment in the pKS$^+$-*prd*0 to produce pKS$^+$-*prd*cDNAp. The sequence between -20 bp from the transcriptional start site and the *Hind*III site in the leader was amplified with the following primers:

prd18bp_FW 5′-ACCGCATCTCGTCCTCGTTCTCG-3′

prd*Hind*III_BW 5′-CGTAAGCTTGTCCCGCTTGTCC-3′

Subsequently, the PCR fragment was digested with *Hind*III and inserted between the *Eco*RV and *Hind*III sites of the pKS$^+$ vector to produce pKS$^+$-*prd*1. To obtain the *prd* construct that includes 20 bp of the upstream region but no intron, the *Not*I-*Hind*III fragment from pKS$^+$-*prd*1 and *Hind*III-*Bsi*WI fragment from pKS$^+$-*prd*cDNA were ligated together into the pW8 vector.

To generate *prd*-mfdelup, first the sequence between -1910 bp and -1270 bp upstream from the transcriptional start site of *prd* was amplified from pKS$^+$-*prd*up by use of the following primers:

prdmfdel1: 5′-CGTATAAATCGCACAATGTGTGC-3′

prdmfdel2: 5′-CCCACAGAGATGTGACGAATGTGC-3′

The PCR fragment was cloned into a *Hind*III-digested and blunt-ended pKS$^+$ vector to produce pKS$^+$-*prd*mfup0. Then the *Not*I-*Dra*III fragment from pKS$^+$-*prd*up was inserted between the *Not*I and *Eco*RV sites of pKS$^+$-*prd*mfup0 to generate pKS$^+$-*prd*mfup1. Smilarly,

the sequence between -234 bp and -660 bp usptream of the transcriptional start site of *prd* was amplified from *prd*-SN20 by use of the following primers:

*prd*mfdel3: 5′-CGAGTGGCAATATAAACGAAAGCC-3′

*prd*mfdel4: 5′-GTGAAAAGCACCGTGGAATGGGAG-3′

The PCR fragment was cloned into a *Hind*III-digested and blunt-ended pKS$^+$ vector to obtain pKS$^+$-*prd*mfup2. The *Pvu*II-*Bsi*WI fragment of *prd*-SN20, including the promoter and transcribed region of *prd* was cloned into the *Eco*RV site of pKS$^+$-*prd*mfup2 to produce pKS$^+$-*prd*mfup3 whereby the correct orientation of the fragment was confirmed by DNA sequencing. The *Not*I-*Kpn*I and *Kpn*I-*Xba*I fragments were excised from pKS$^+$-*prd*mfup2 and pKS$^+$-*prd*mfup3, respectively, and cloned into the pW8 vector to obtain the *prd*-mfdelup construct.

The *prd-Gal4::VP16* fusion constructs were produced as follows. First, the sequences including the *prd* promoter, leader, and the coding region of *prd* encoding the N-terminal paired-domain and part of the homeodomain, were amplified from *prd*-SN20 by the use of the following primers:

prdupstart: 5′- GAAAAGAGGCTACTCGAGTGGC-3′

RevjuncprdupGal4: 5′-GTAGCTTCATCTGGCCGCTGTTCATGTCT-3′

The second fragment, including the *Gal4::VP16* sequences, was amplified by the use of the following primers:

ForprdUjunGal4: 5′-CAGCGGCCAGATGAAGCTACTGTCTTCTAT-3′

RevGal4junprdD: 5′-CTCTTTGATTCTACCCACCGTACTCGTCAA-3′

The third fragment, including the stop codon and the trailer sequences of *prd*, was amplified by use of the following primers:

ForGal4junprdD: 5′-CGGTGGGTAGAATCAAAGAGACACGGATCC-3′

prdstop+trailer: 5′-TTCCGCTCGTAGCTTTGCTGGG-3′

All three PCR fragments were denatured and allowed to anneal through overlapping sequences at their ends and then the full length PCR fragment, consists of *prd* promoter, the leader, part of the *prd* coding region fused to *Gal4::VP16* coding region in frame, and the trailer of *prd*, was amplified using the terminal primers:

prdupstart: 5′- GAAAAGAGGCTACTCGAGTGGC-3′

prdstop+trailer: 5′-TTCCGCTCGTAGCTTTGCTGGG-3′

This PCR fragment was cloned into the *Eco*RV site of the pKS$^+$ vector to generate *prd*1-2Gal4*::*Vp16*prd*0. The correct orientation of the fragment was confirmed by sequencing.

Subsequently, the cloned PCR fragment was excised as *Not*I-*Xba*I fragment from *prd*1-2Gal4*::*Vp16*prd*0 and inserted between the *Not*I and *Xba*I sites of the pW8 vector to produce *prdupsnew0-Gal4::VP16*.

To generate the *prdupsnew(1-4)-Gal4::VP16* constructs (Figs. 9A and 10A), different upstream *cis*-regulatory regions of *prd* were amplified from pKS$^{+}$-*prd*up by PCR. The upstream sequence between 7.6 kb and 5.6 kb from the transcriptional start site of *prd* was amplified by the use of the following primers, including a *Not*I restriction site (underlined), digested with *Not*I, and cloned into the *Not*I site of *prdupsnew0-Gal4::VP16* to produce *prdupsnew1-Gal4::VP16*:

prdupsnew1_FW 5′-ATCAATGCGGCCGCTTGTGGACTACACATAAGTGTG-3′

prdupsnew1_BW 5′-ATCAATGCGGCCGCGGAAAAGTGAGCGTTTTCCAGG-3′

The upstream sequence between 5.9 kb and 3.8 kb from the transcriptional start site of *prd* was amplified by the use of the following primers, including a *Not*I restriction site (underlined), digested with *Not*I, and cloned into the *Not*I site of *prdupsnew0-Gal4::VP16* to produce *prdupsnew2-Gal4::VP16*:

prdupsnew2_FW 5′-ATCAATGCGGCCGCATATTGTGGCTGGTGTCGTGTG-3′

prdupsnew2_BW 5′-ATCAATGCGGCCGCTCGCAATTGGTGGAAACTTCGC-3′

The upstream sequence between 4.2 kb and 2.0 kb from the transcriptional start site of *prd* was amplified by the use of the following primers, including a *Not*I restriction site (underlined), digested with *Not*I, and cloned into the *Not*I site of *prdupsnew0-Gal4::VP16* to produce *prdupsnew3-Gal4::VP16*:

prdupsnew3_FW 5′-ATCAATGCGGCCGCCATGATCGGCGAAAATTCAGCC-3′

prdupsnew3_BW 5′-ATCAATGCGGCCGCGCTGTTGCTTTTATACTACGCG-3′

The upstream sequence between 2.4 kb and 234 bp from the transcriptional start site of *prd* was amplified by the use of the following primers, including a *Not*I restriction site (underlined), digested with *Not*I, and cloned into the *Not*I site of *prdupsnew0-Gal4::VP16* to produce *prdupsnew4-Gal4::VP16*:

prdupsnew4_FW 5′-ATCAATGCGGCCGCTTATTGCACTGCAATGGCATGC-3′

prdupsnew4_BW 5′-ATCAATGCGGCCGCCGCGTTTAACACTCTTTACAAGC-3′

**Generation of transgenic flies**

Constructs were injected together with pUChspΔ2-3 P-element helper plasmid (D. Rio, personal communication) into *y w* embryos according to standard procedures and $w^{+}$ transformants were selected. Transgenic *prd*$^{-}$ embryos carrying one or two copies of the

specified transgenes were obtained as follows. Two types of stocks were established for all transgenes, *y w*; $prd^{2.45}$/*CyO*; P/P and *y w*; $prd^{32.12}$/*CyO*; P/P (P stands for the $w^+$ P elements that contain the transgenes). To analyze the larval cuticular phenotype or to measure the survival to adulthood of *prd* mutants with one or two copies of the transgenes, the offspring were collected from crosses between *y w*; $prd^{2.45}$/*CyO*; P/P and *y w*; $prd^{32.12}$/*CyO* or between *y w*; $prd^{2.45}$/*CyO*; P/P and *y w*; $prd^{32.12}$/*CyO*; P/P. To test the functional significance of the accessory gland enhancer in the upstream region of *prd*, $prd^{2.45}$/*CyO*; *prd*-mfdelup/*prd*-mfdelup flies were crossed with $prd^{32.12}$/*CyO*; *prd*-mf5/*prd*-mf5 flies, as a *prd* transgene under the sole control of the upstream region, such as *prd*Res, is not sufficient to initiate development of accessory glands (Xue and Noll, 2002).

**Immunostaining of embryos, and preparations of accessory glands and larval cuticle**

Immunostaining of embryos with anti-Prd or anti-ß-galactosidase was carried out as described (Gutjahr et al., 1994). Accessory glands were dissected in 1x Ringer's solution and stained with anti-Prd or anti-ß-galactosidase antibodies (Xue and Noll, 2002). Embryos were collected on agar plates and allowed to develop for 24 hours at 25°C before preparation of cuticles, essentially as described (Wieschaus and Nüsslein-Volhard, 1986).

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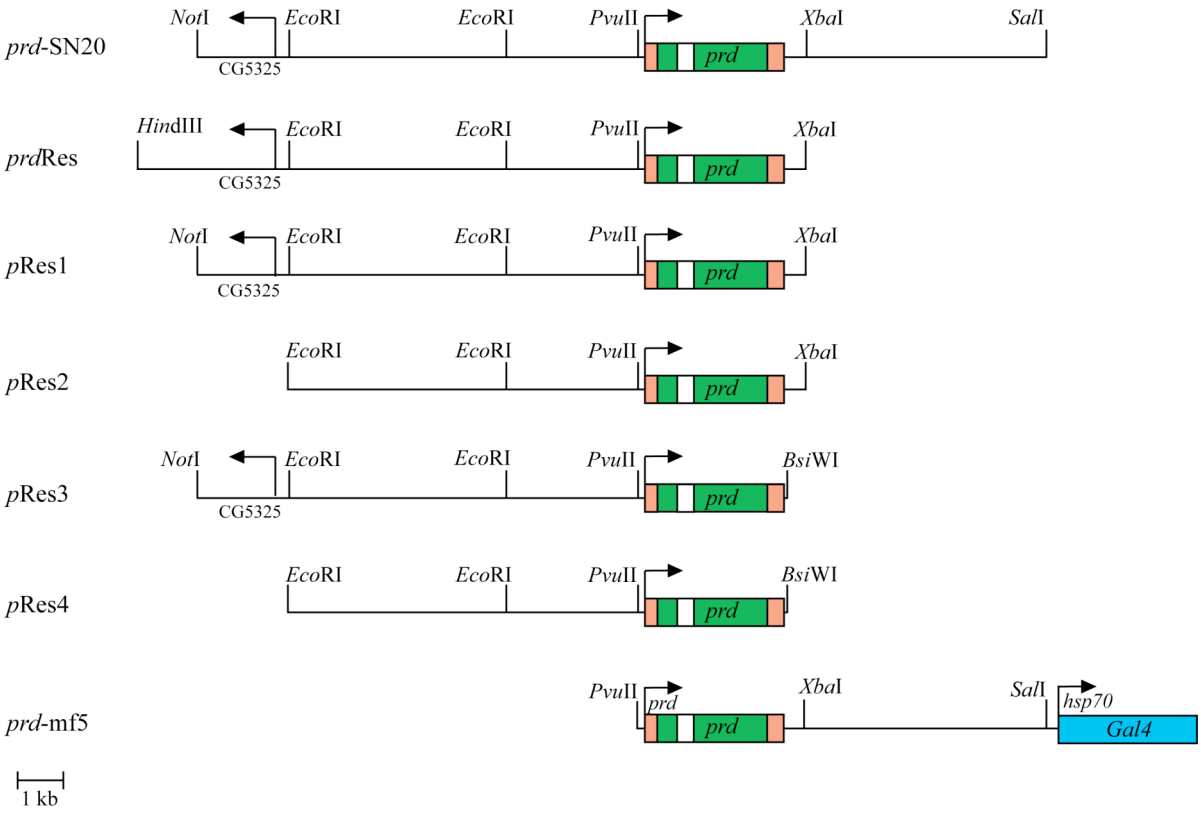


**Figure 1. Map of *prd* transgenes.** The *prd*-SN20 transgene is an 18 kb genomic fragment that includes the entire *prd* gene, consisting of its transcribed region as well as as of the adjacent 9.7 kb upstream and 5.7 kb downstream sequences (Gutjahr et al., 1994). The *prd*Res transgene includes 11 kb upstream and only 0.5 kb of the downstream region (Bertuccioli et al., 1996). These transgenes also contain part of the neighboring gene, CG5325. The transcriptional start of the neighboring gene is marked and its direction of transcription indicated by an arrow. The *prd*-mf5 transgene is a *prd-Gal4* transgene consisting of the *prd* promoter, *prd* transcribed region, and 5.7 kb adjacent downstream sequences placed upstream of the *hsp70* basal promoter and the *Gal4* gene. All other transgenes are described in the text.

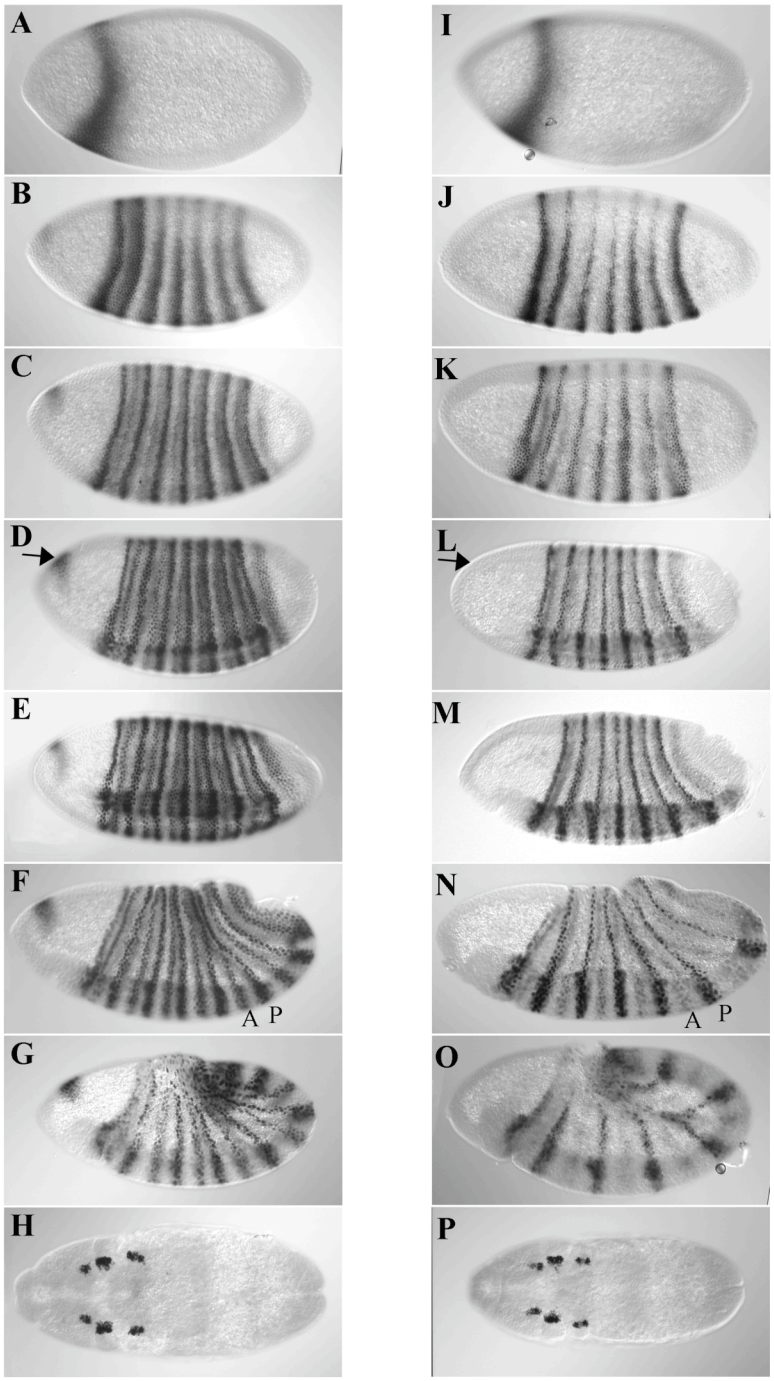


**Figure 2. Expression patterns of *prd*Res transgene.** Expression of Prd protein in wild-type (A-H) and homozygous $prd^{2.45}$ embryos carrying two copies of *prd*Res transgene (I-P). $w^{1118}$; $prd^{2.45}$; *prd*Res embryos were collected from a $w^{1118}$; $prd^{2.45}$/*CyO hb-lacZ*; *prd*Res stock whose *CyO* chromosome expresses ß-galactosidase under the control of the *hunchback* (*hb*) promoter. Anterior (A) and posterior (P) stripes of Prd protein are identified (F, N), and the arrow indicates the position of dorsal spot (D, L).

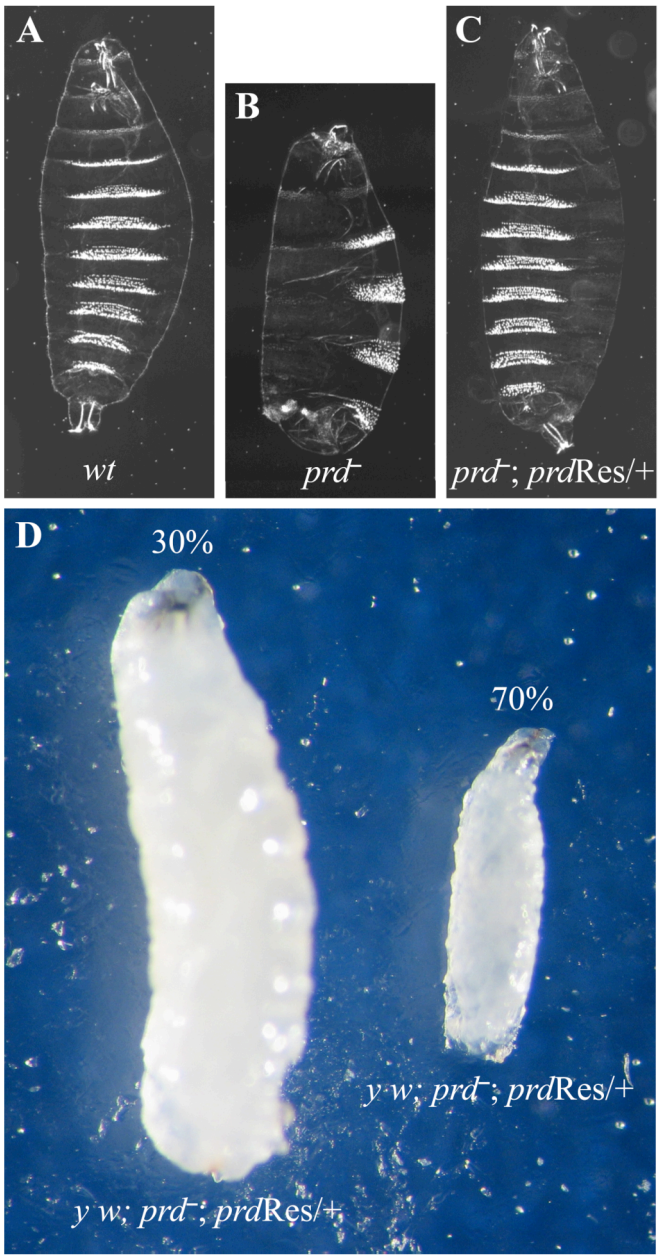


**Figure 3**. **Complete rescue of cuticular phenotype of *prd* embryos by one copy of *prd*Res transgene.** Ventral views of cuticles of wild-type (A), $w^{1118}$; $prd^{2.45}/prd^{32.12}$ embryos (B), and $w^{1118}$; $prd^{2.45}/prd^{32.12}$ embryos carrying one copy of *prd*Res (C) are shown under dark-field illumination (anterior is up). Embryos were collected and allowed to develop for 24 hours at 25°C before cuticle preparation. (D) Larval phenotype of *y w*; $prd^{2.45}/prd^{32.12}$ mutants rescued with one copy of *prd*Res transgene.

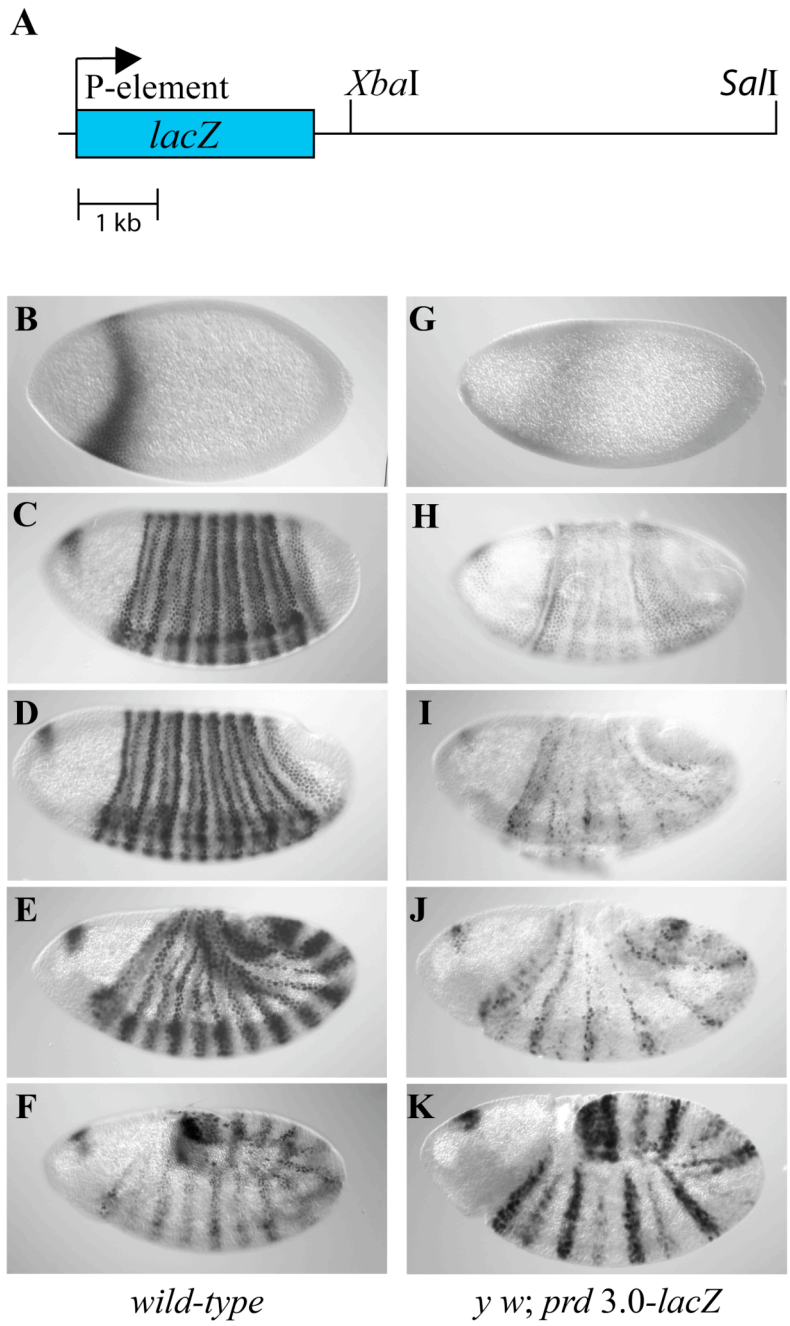


**Figure 4. Downstream enhancers of *prd* control expression in stripes and in the dorsal spot.** (A) Schematic view of the *prd* 3.0-*lacZ* construct, a *lacZ* reporter transgene expressed under the control of the 5.7 kb *prd* downstream region and the P-element promoter. Expression patterns of *prd* in wild-type embryos (B-F) and of *lacZ* in wild-type embryos carrying the *prd* 3.0-*lacZ* transgene (G-K).

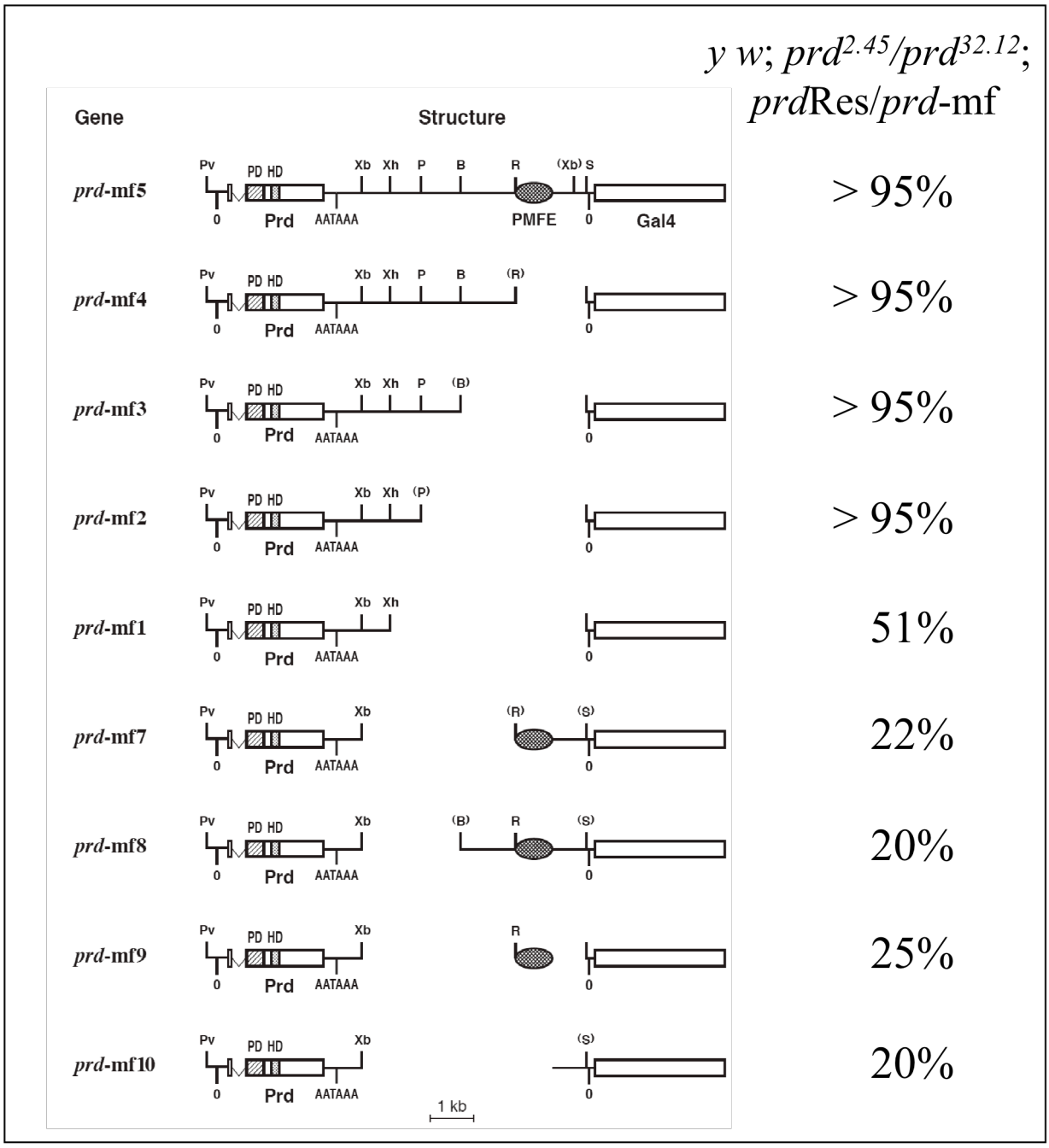


**Figure 5. Mapping of the survival enhancer in the downstream region of *prd*.** Schematic representation of *prd*-mf transgenes consisting of the *prd* promoter, *prd* transcribed region, adjacent downstream sequences of *prd*, the *hsp70* basal promoter, and the yeast *Gal4*-coding region. One copy each of *prd*Res and *prd*-mf transgenes were used to rescue transheterozygous *prd* null mutants (*y w*; $prd^{2.45}/prd^{32.12}$) to adulthood. The *prd*-mf5, *prd*-mf4, *prd*-mf3, and *prd*-mf2 transgenes rescue more than 95% of the *prd* mutans, while *prd*-mf1 rescues only 50% to adulthood. By contrast, *prd*-mf7, *prd*-mf8, *prd*-mf9, and *prd*-mf10 do not enhance the survival efficiency of the *prd*Res transgene.

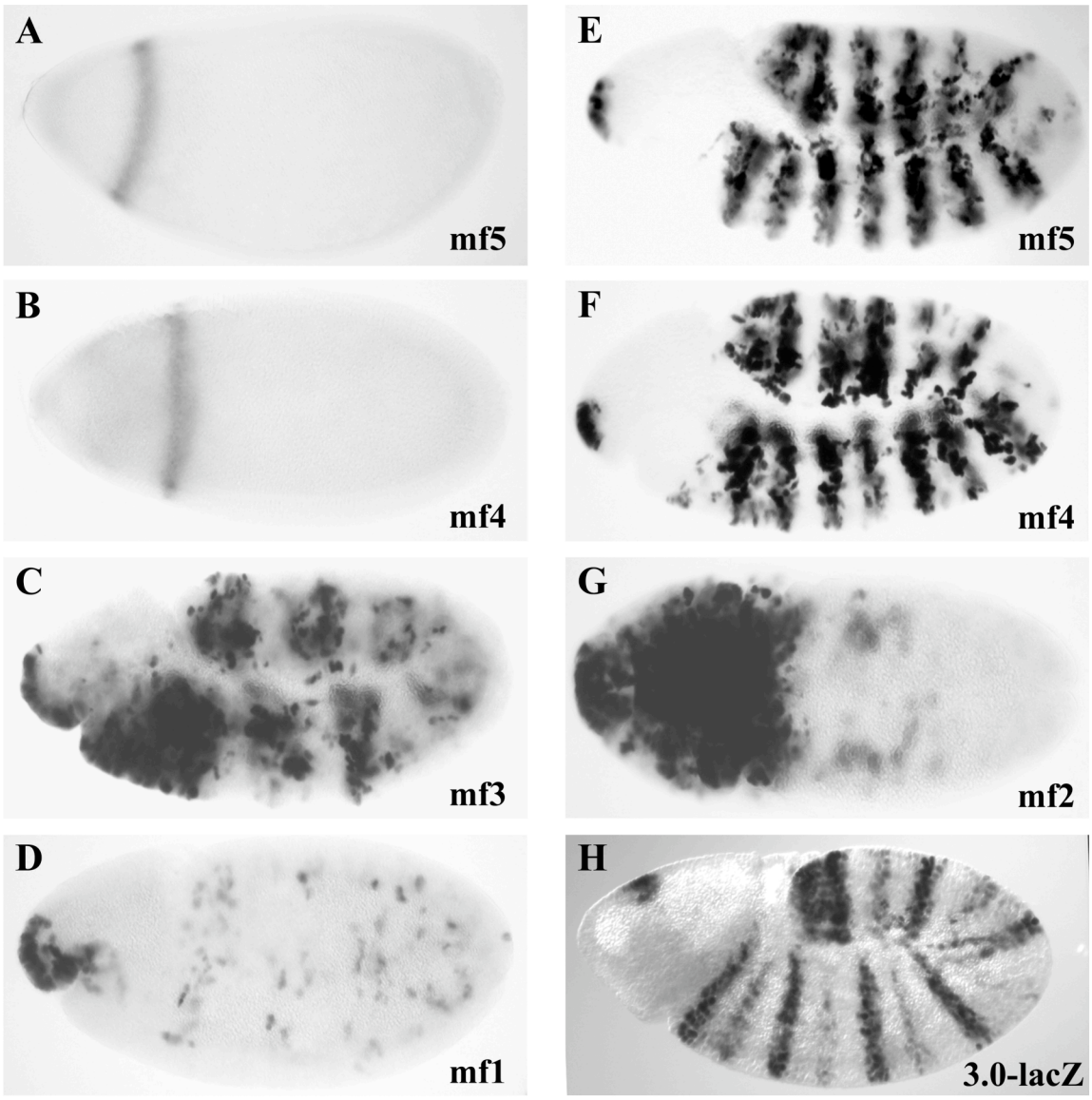


**Figure 6. Mapping of embryonic enhancers in the downstream region of *prd*.** Expression of Gal4 (A-G) or LacZ (H) in embryos carrying two copies each of UAS-GFP and *prd*-mf5 (A, E), *prd*-mf4 (B, F), *prd*-mf3 (C), *prd*-mf2 (G), or *prd*-mf1 (D), or two copies of *prd* 3.0-*lac*Z (H) at syncytial blastoderm (A, B), during germ band extension (E, F, H), or at the extended germ band stage (C, D, G). Embryos are shown as lateral (A, C-F, H) or dorsal (B, G) views and are oriented with their anterior to the left. Embryos were stained immunohistochemically with antibodies against GFP (A-G) or β-galactosidase (H).

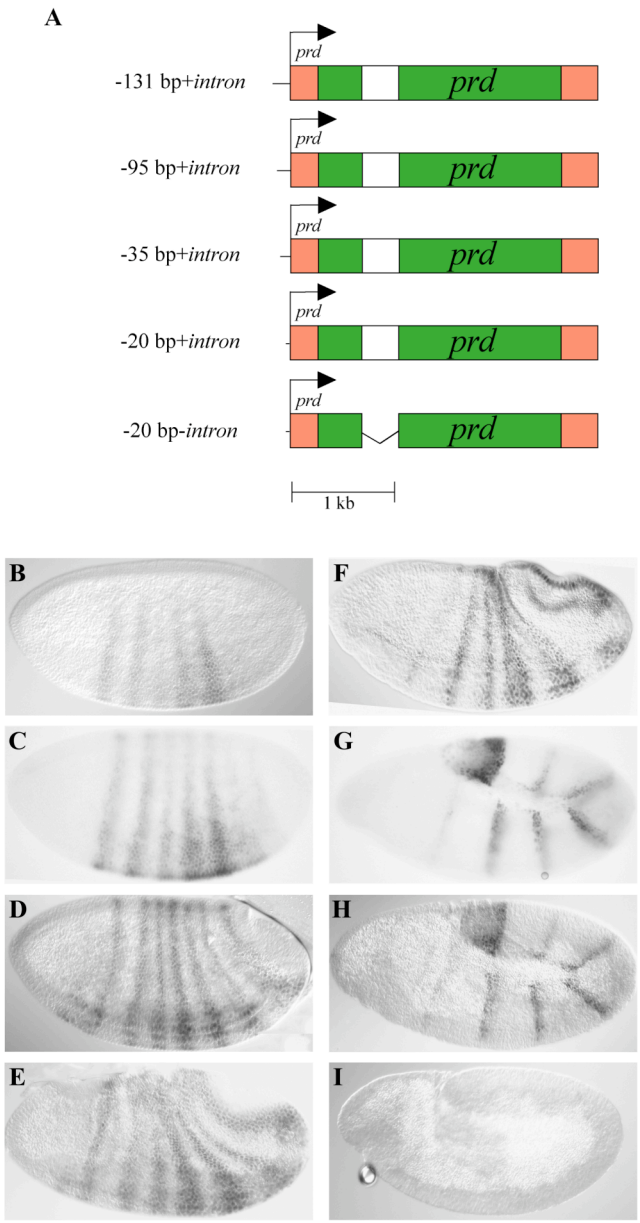


**Figure 7. The intron of *prd* activates a weak pair-rule expression pattern in the embryo.** (A) Map of minimal *prd* transgenes, consisting of the coding region (green), 5' and 3' untranslated regions (saffron), intron (white), and different short upstream regions (black line) of *prd*. (B-H) Expression of Prd in *y w*; $prd^{2.45}$ embryos, carrying a *prd* transgene including 35 bp (B, C, F, G) or 20 bp (D, E, H) of upstream region and the *prd* intron, during nuclear cycle 14 of syncytial blastoderm (B, C), at early gastrulation (D), at mid-gastrulation (E), at the onset of (F) and during germ band extension (G, H). (I) Expression of Prd in *prd* null mutant embryos, carrying a *prd* transgene including 20 bp of upstream region but no intron, during germ band extension. Both transgenes including the *prd* intron show the same weak expression pattern of 7 pair-rule stripes, presumably corresponding to narrow stripes 2-7 and a broad stripe 8 (B-H), whereas no expression is apparent in the absence of the intron (I). All the embryos are oriented with their anterior to the left and dorsal side up.

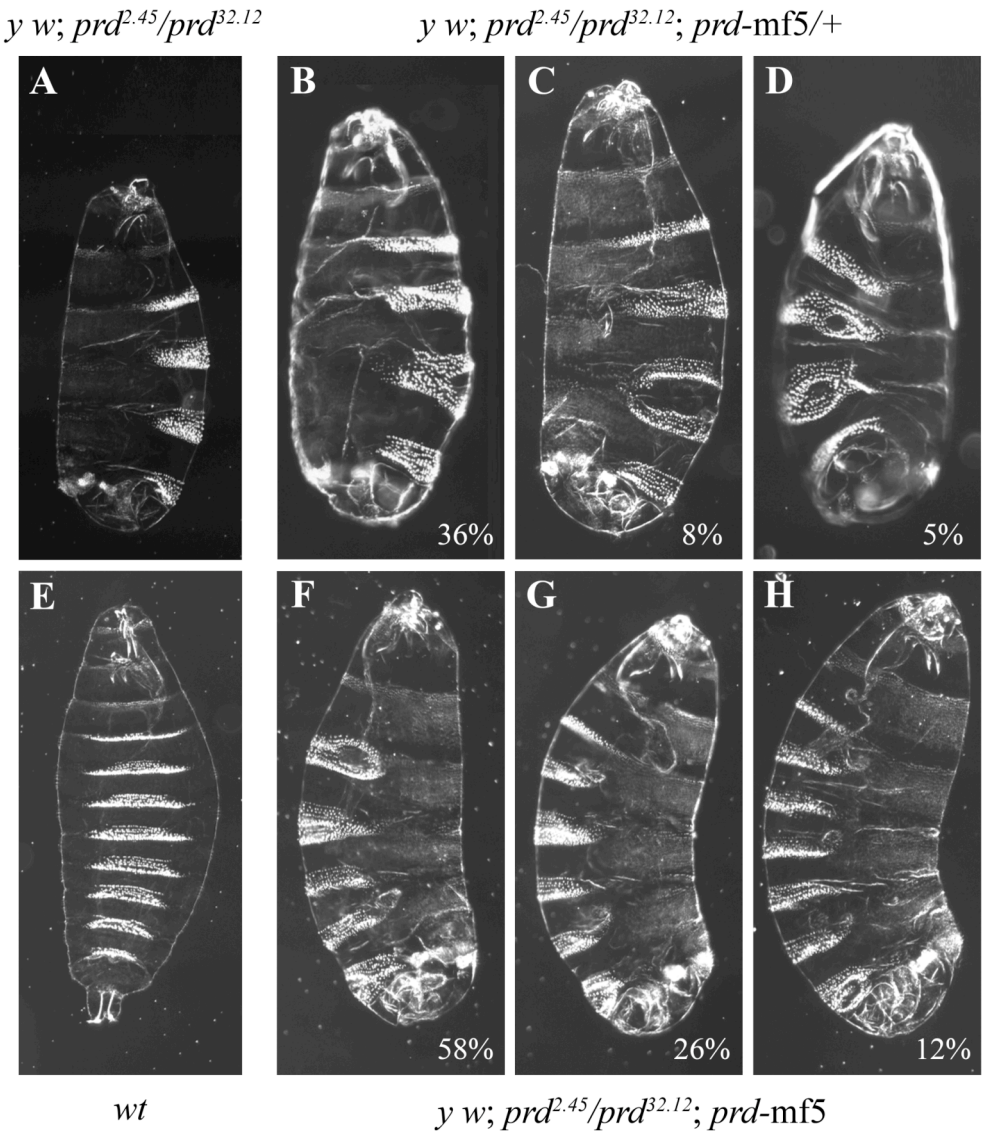


**Figure 8. Rescue of cuticular phenotype of *prd*⁻ embryos by *prd*-mf5 transgene.** Lateral views of cuticle preparations of *y w*; $prd^{2.45}/prd^{32.12}$ embryos (A), *y w*; $prd^{2.45}/prd^{32.12}$ embryos rescued by one (B-D) or two copies of *prd*-mf5 (F-H) are shown under dark-field illumination (anterior is up). For comparison, a ventral view of a wild-type embryo is shown in (E) at a slightly lower magnification. *prd*⁻ embryos show the classical pair-rule phenotype, in which every other segment is missing (A). One copy of *prd*-mf5 shows very little rescue of the *prd*⁻ cuticular phenotype, whereby frequently a partial rescue of one segment (B and C) and rarely of two segments (D) is observed. Two copies of *prd*-mf5 achieve a better rescue of the *prd*⁻ cuticular phenotype, except in the posterior abdominal segments where rescue is always incomplete (F-H).

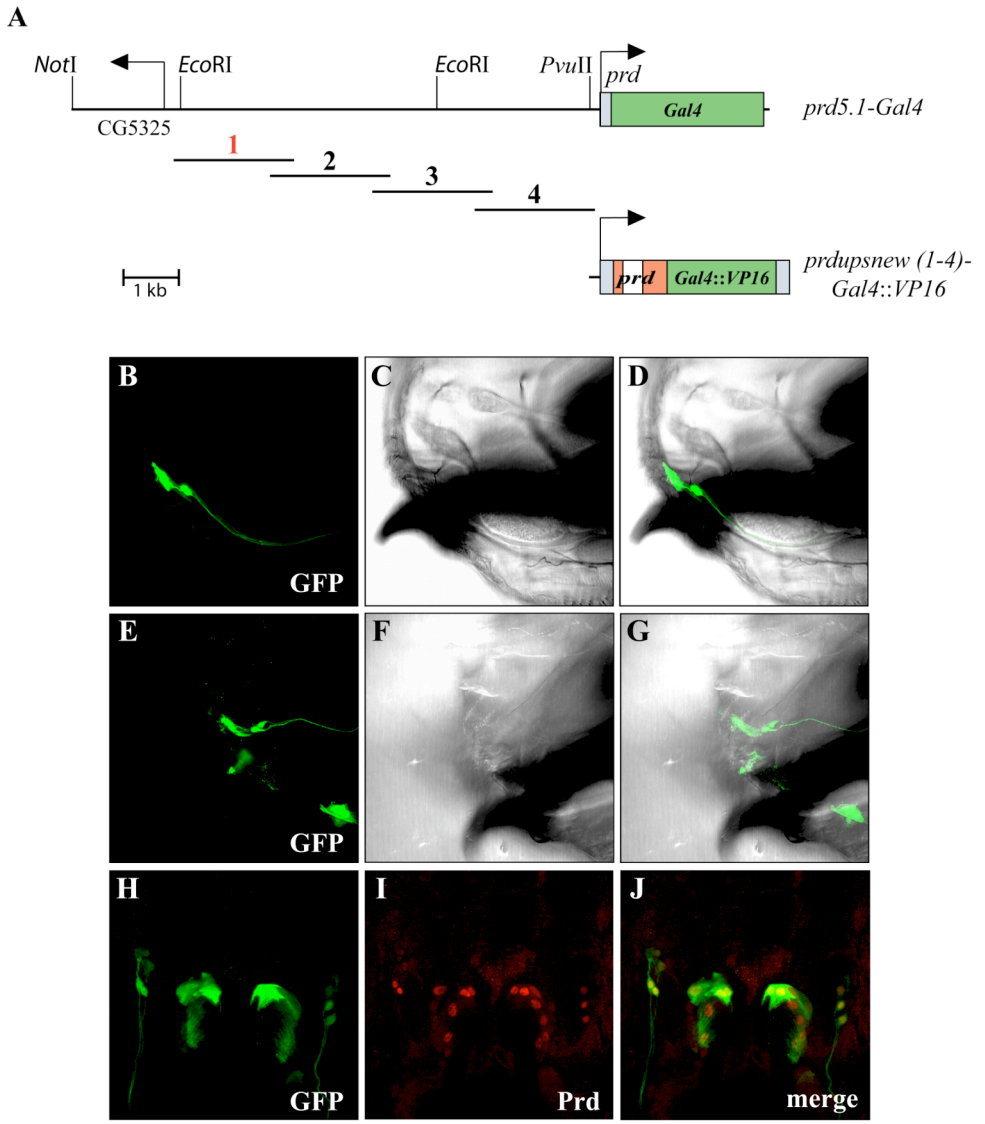


**Figure 9. *prd* expression in the larval ventral organ under control of a distal upstream enhancer.** (A) Maps of *prd5.1-Gal4* transgene (L. Xue and M. Noll, unpublished results) and of a series of *prd-Gal4::VP16* transgenes under the control of different overlapping upstream fragments, labeled 1-4, and the *prd* promoter. These upstream fragments were fused to a *prd* fragment extending from the *prd* promoter to the second exon (encoding the paired-domain and part of the homeodomain), which was fused in frame to a fragment encoding the VP16 activating domain, followed by the *prd* trailer. (B-D) Expression of GFP driven by *prd5.1-Gal4* in the ventral organ. (E-G) Expression of GFP driven by *prd-Gal4::VP16* transgene under the control of the *prd* upstream fragment 1 (A) in the ventral organ of a third instar larva. (H-J) Expression of Prd in the ventral organ was confirmed by double-staining for Prd (I, J) and GFP (H, J), whose expression was driven by *prd5.1-Gal4*, by the use of anti-Prd and anti-GFP antisera. Pictures in (A-G) were taken at 40x, those in (H-J) at 63x magnification.

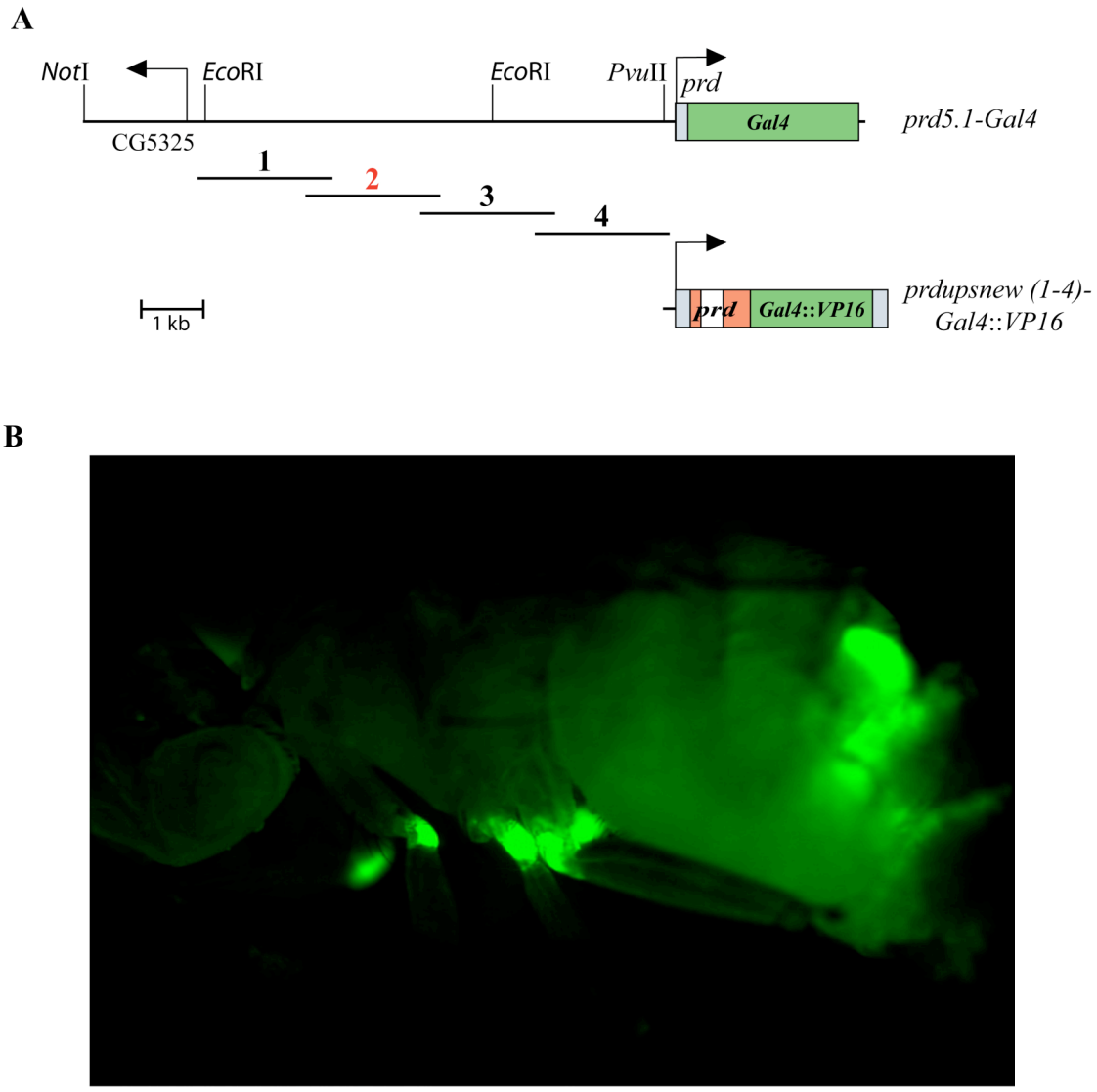


**Figure 10. Mapping an upstream enhancer regulating *prd* expression in the trochanter segment of legs.** (A) Maps of *prd5.1-Gal4* and a series of *prd-Gal4::VP16* transgenes under the control of different overlapping upstream fragments, labeled 1-4, and the *prd* promoter (cf. legend to Fig. 9A). (B) Expression of GFP in the trochanter of a freshly eclosed fly, driven by a *prd-Gal4::VP16* transgene under the control of fragment 2, located between 5.7 kb and 3.5 kb upstream of the transcriptional start of *prd*.

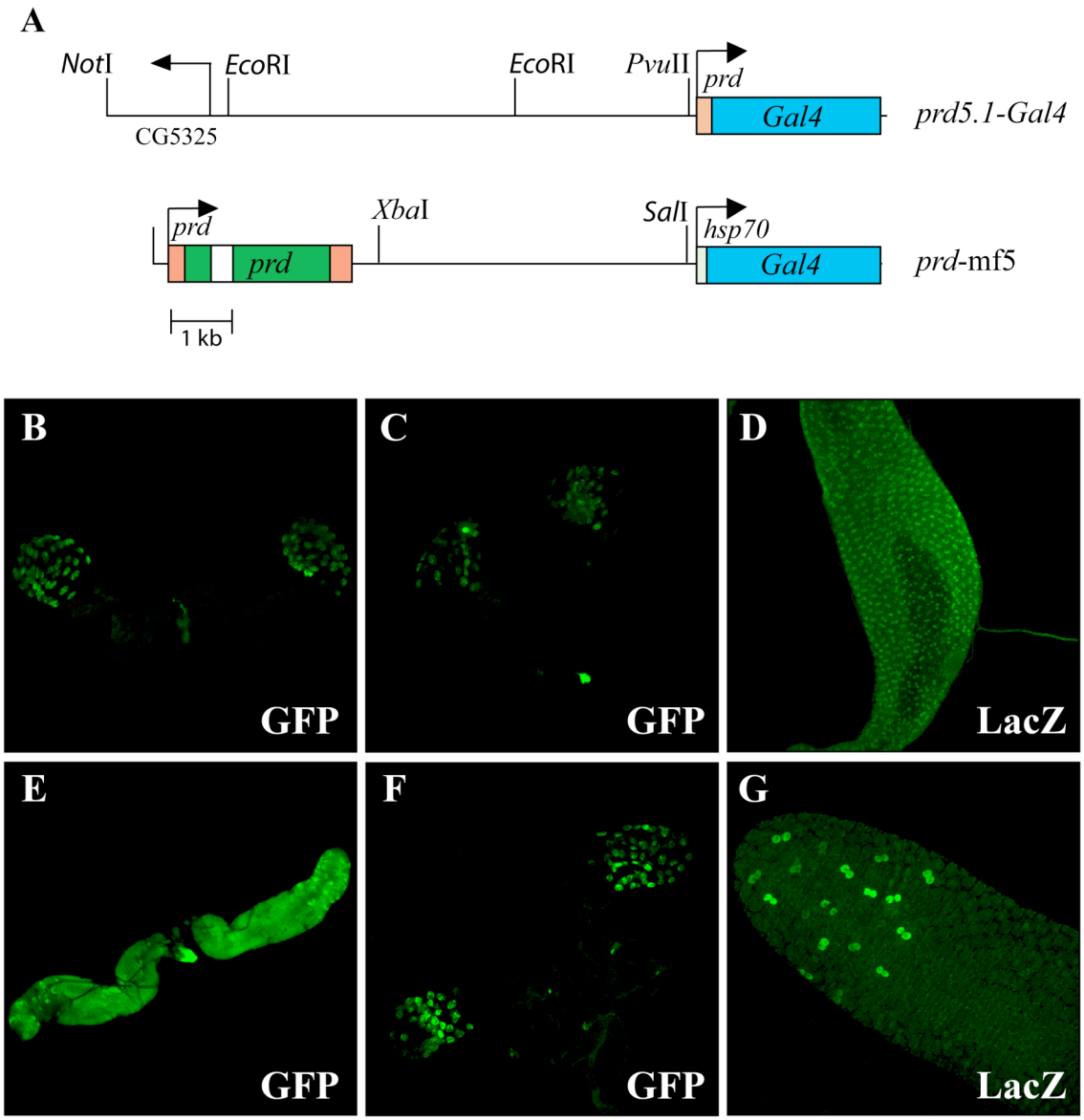


**Figure 11. An upstream enhancer of *prd* regulating transcription in accessory glands.** (A) Maps of *prd5.1-Gal4* (s. legend to Fig. 9A) and *prd*-mf5 transgenes. In the latter, *prd* is under the control of the *prd* promoter and the entire 5.7-kb downstream region of *prd*, which also regulates *Gal4* through an *hsp70* basal promoter (Xue and Noll, 2002). (B, C, E, F) Expression of UAS-GFP driven by *prd*-mf5 (B, C) or *prd5.1-Gal4* (E, F) in accessory glands of one day-old (B, E) or 5 day-old (C, F) virgin males. (D, G) Expression of a *lacZ* reporter gene, *prdΔC*, under control of the complete upstream *cis*-regulatory region of *prd* (Figure 12A; Gutjahr et al., 1994), in accessory glands of one day-old (D) or 5 day-old (G) virgin males, stained with an anti-b-galactosidase antiserum. Glands were dissected in 1xRinger's solution and pictures were taken under a Leica TCS SP confocal microscope at 10x (C, E, F), 20x (B, D), or 40x (G) magnifications.

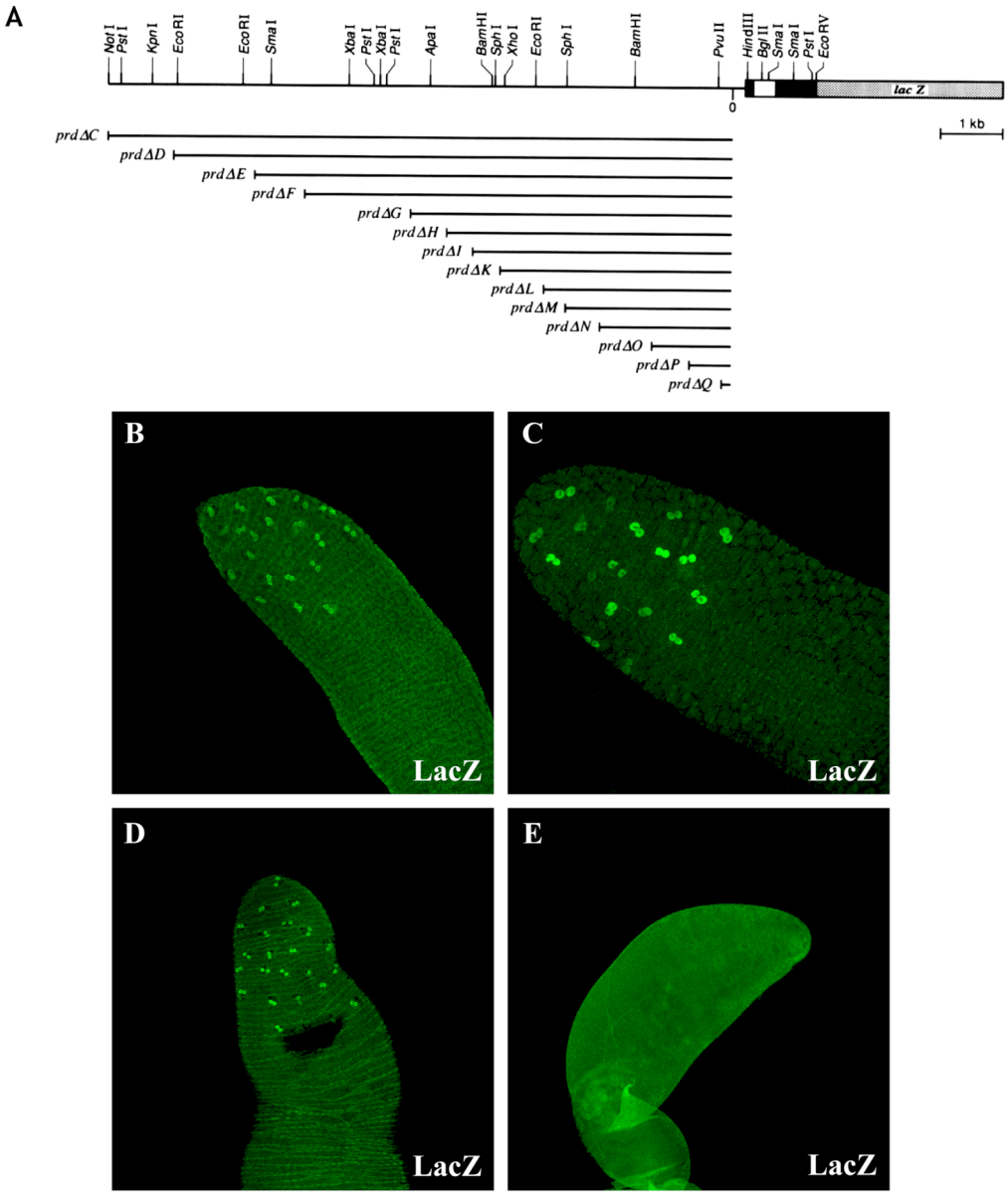


**Figure 12. Mapping of accessory gland enhancer to a proximal upstream region of *prd*.** Map of *lacZ* reporter constructs under the control of decreasing lengths of the *prd* upstream region and the *prd* promoter, in which the *lacZ* coding region was fused in frame to the *prd* coding region, including the paired-domain and part of the homeodomain as indicated (Gutjahr et al., 1994). (B-E) Expression of fusion protein by *prd*Δ*C* (B), *prd*Δ*M* (C), *prd*Δ*O* (D), and *prd*Δ*P* (E) in accessory glands of 4-day old virgin males. The *prd*Δ*O* transgene is expressed in accessory glands, whereas *prd*Δ*P* is not, which maps an essential part of the enhancer to the upstream region between ΔO and ΔP. Glands were dissected in 1xRinger's solution and stained with anti-β-galactosidase antibody. Pictures were taken under a Leica TCS SP confocal microscope at 40x magnification.

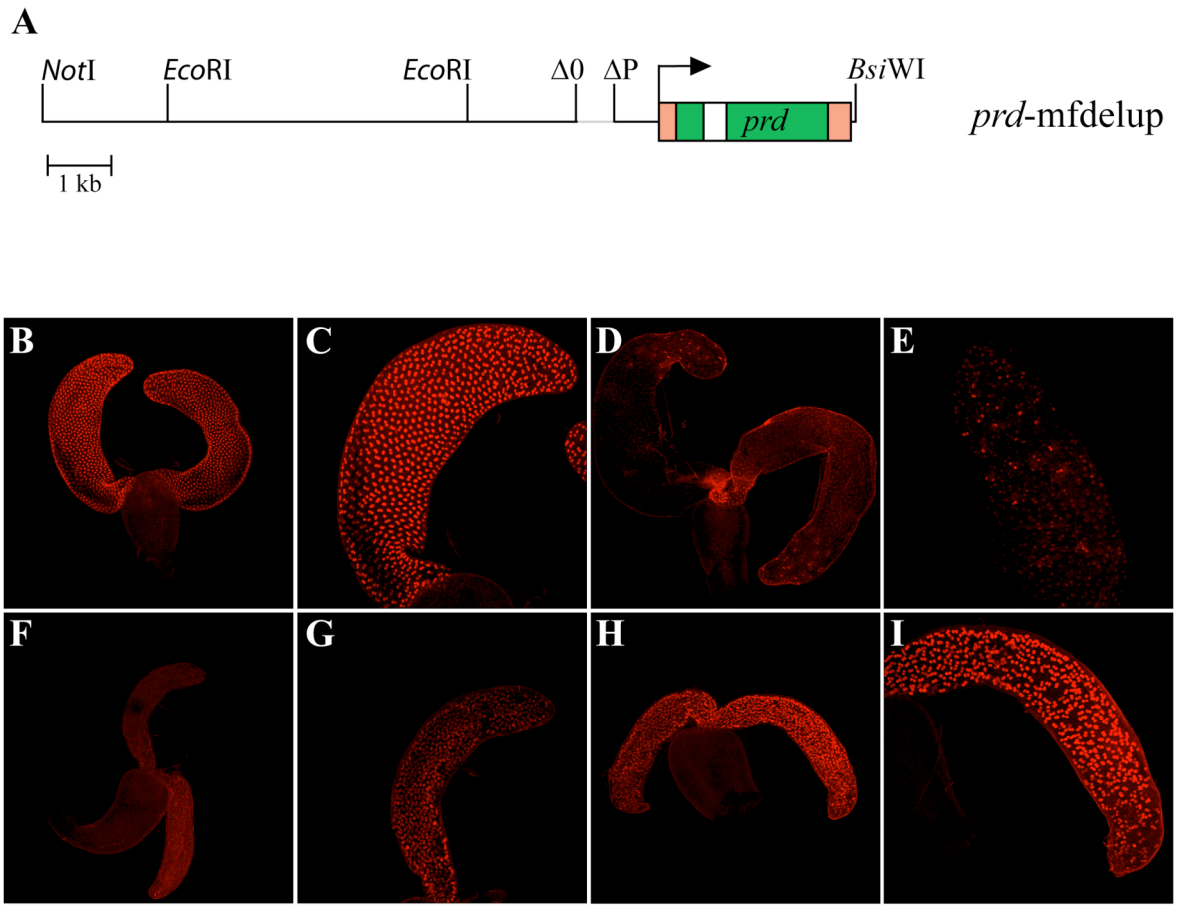


**Figure 13. The accessory gland enhancer in the upstream region of *prd* is required for its male fertility function.** (A) Map of *prd*-mfdelup transgene used for partial rescue of *prd* mutants to adulthood. It carries a deletion between ΔO and ΔP of the upstream region, which includes essential sequences of the accessory gland enhancer. (B-E) Prd expression in accessory glands of 1-day old (B, C) or 5-day old (D, E) wild-type virgin males. (F-I) Prd expression in accessory glands of 1-day old (F, G) or 5-day old (H, I) *y w*; $prd^{2.45}/prd^{32.12}$ virgin males rescued by one copy each of *prd*-mfdelup and *prd*-mf5. The rescued *y w*; $prd^{2.45}/prd^{32.12}$ males are sterile. Prd protein is made visible by immunohisto-chemical staining with an anti-Prd antiserum. Pictures were taken under a Leica TCS SP confocal microscope at 10x (D), 20x (B, E, F, H), or 40x (C, G, I) magnification.

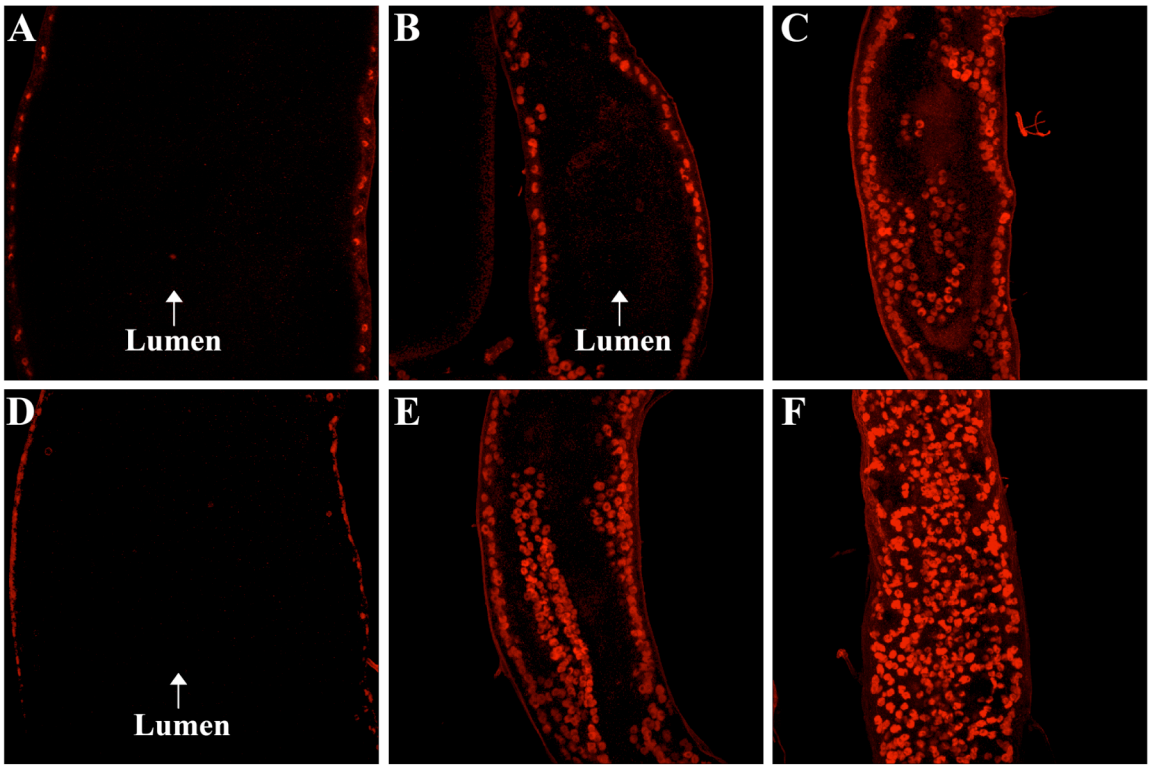


**Figure 14. Defect in maintaince of accessory gland lumen in the absence of the upstream accessory gland enhancer of *prd*.** Confocal sections of accessory glands of 1-day old (A) and 5-day old (D) wild-type virgin males, and of 1-day old (B), 2-day old (C, E), and 5-day old (F) *y w*; $prd^{2.45}$/$prd^{32.12}$; *prd*-mfdelup/*prd*-mf5 virgin males. The accessory gland lumen of the rescued mutant virgin males begins to collaps by 2 days (C, E, F). The glands were dissected in 1x Ringer's solution and stained for Prd protein with an anti-Prd antiserum. All pictures were taken under a Leica TCS SP confocal microscope at 40x magnification.

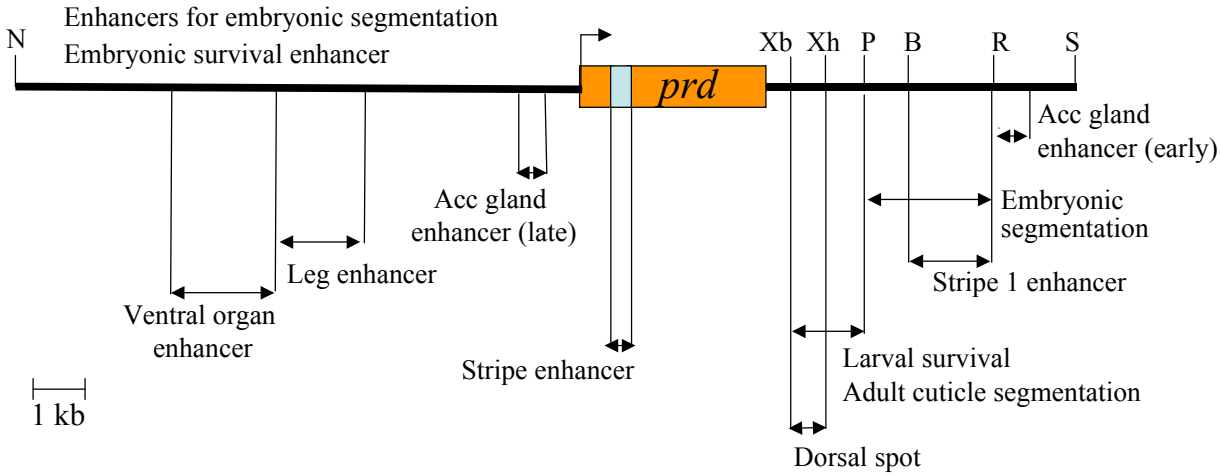


**Figure 15. Map of enhancers regulating the specific functions of the *prd* gene and defined in this thesis.** The upstream region of *prd* has been shown to contain enhancers for embryonic segmentation and survival of *prd* mutants to adulthood (Gutjahr., 1994; Xue et al., 2001). The accessory gland enhancer (early) has been mapped to a 500 bp region in the distal downstream region of *prd* (Xue and Noll, 2002). In this study, the enhancers controlling *prd* expression in the ventral organ and leg (trochanter) were mapped in the upstream region. An enhancer for late expression of *prd* in accessory glands was also mapped in the upstream region and shown to be required for the male fertility function of *prd*. Intronic sequences of *prd* were shown to produce a stripe pattern in the embryo. The downstream region exhibits a complex organization of enhancers required for embryonic expression, survival to adulthood, and proper segmentation of the adult cutile. This last enhancer is characterized in Chapter 3.

| Rescue construct | Rescue with one copy | Rescue with two copies |
|---|---|---|
| $prd^{2.45}/prd^{32.12}$; *prd*-SN20 | > 95% | > 95% |
| $prd^{2.45}/prd^{32.12}$; *prd*Res | **20%** | > 90% |
| $prd^{2.45}/prd^{32.12}$; *prd*Res/*prd*-mf5 | > 95% | nd |
| $prd^{2.45}/prd^{32.12}$; *p*Res1 | **22%** | 82% |
| $prd^{2.45}/prd^{32.12}$; *p*Res2 | **24%** | 78% |
| $prd^{2.45}/prd^{32.12}$; *p*Res3 | **20%** | 85% |
| $prd^{2.45}/prd^{32.12}$; *p*Res4 | **20%** | 74% |
| $prd^{2.45}/prd^{32.12}$; *prd*-mf5 | die as embryos | die as embryos |
| $prd^{IIB42}/prd^{2.45}$; *prd*Res/+ | > 95% | nd |
| $prd^{IIN27}/prd^{2.45}$; *prd*Res/+ | 50% | nd |
| $prd^{IIN27}/prd^{IIB42}$; *prd*-mf5 | No rescue | No rescue |

**Table 1. Rescue of *prd* mutants to viable adults by one or two copies of *prd* transgenes.** The rescue efficiency of *prd* transgenes is shown as percentage of $prd^-$ embryos rescued to adulthood. Transgenic embryos were produced by the crosses described in Materials and Methods. $prd^{2.45}$ and $prd^{32.12}$ are null alleles of *prd*. $prd^{IIB42}$ is a strong hypomorphic allele, while $prd^{IIN27}$ is a weak hypomorphic allele missing the C-terminal portion of the Prd protein (Xue et al., 2001).

# Chapter 3

# Role of *prd* in abdominal segmentation of adult cuticle

## Summary

Rescue experiments of *prd* mutants with the evolutionary alleles of *prd*, *prd*-Gsb and *prd*-Pax3, uncovered a novel function of *prd* in adult cuticle segmentation. In this study, we investigated the adult cuticle function of *prd* using partial rescue transgenes of *prd*. The *prd*Res transgene, which lacks almost the entire downstream *cis*-regulatory region, rescues as a single copy only 20% of *prd* mutants to adulthood, and the rescued flies exhibit partial fusions of abdominal segments. Two copies of *prd*Res rescue this adult cuticle function to almost wild-type. One copy each of *prd*Res and *prd*-mf5 transgenes rescue the adult abdominal segmentation phenotype completely. However, the downstream enhancers alone are not sufficient to rescue the adult cuticle function of *prd*. The conditional knockout of *prd* during post-embryonic stages revealed a crucial role of *prd* in the development of the adult abdominal segments. The knockout of *prd* in the histoblasts produces a severe disorganization of abdominal segments, which demonstrates that *prd* functions in the histoblasts to control the development of abdominal segments.

## Introduction

The insect segment is an excellent model for studying the molecular mechanisms regulating patterning and cell polarity. Extensive genetic and molecular analysis, using *Drosophila* as a model organism, showed that cells within a segment decide when to undergo cell division or cell death, differentiate into distinct structures, and how to organize these structures based on signals received from other cells in the same segment or from the cells in a neighbouring segment (reviewed by Lawrence, 1992; Struhl et al., 1997). Each epidermal segment in *Drosophila* is subdivided into an anterior and posterior compartment that are defined by the expression of selector genes (Lawrence and Struhl, 1996). The study of mitotic clones in wing discs have led to a general model of how compartments and selector genes regulate pattern. Compartment boundaries are determined by the selector gene *engrailed* (*en*), which is expressed in the posterior but not the anterior compartment of each segment. Pattern formation within each segment is largely dependent on cell-cell interactions at compartment boundaries (Tabata et al., 1995; reviewed by Lawrence and Struhl, 1996). Posterior cells under the control of the *en* gene secrete a short-range morphogen, Hedgehog (Hh). Hh crosses the compartment boundary and activates long-range morphogens, the products of *decapentaplegic* (*dpp*) or *wingless* (*wg*), in the very adjacent anterior cells at the compartment boundary (Nellen et al., 1996). The long-range morphogens regulate the pattern of both anterior and posterior compartments. Wg plays such a role in the embryonic epidermis (Dougan and DiNardo, 1992; Lawrence et al., 1996), and Dpp has been shown to pattern both the anterior and the posterior compartment of the wing disc (de Celis et al., 1996; Lecuit et al., 1996; Nellen et al., 1996).

The larval cuticle is secreted by large, polyploid cells derived from the epidermal cells of the mature embryo. Unlike head and thorax of the adult fly, the cuticle of the adult abdomen is not produced by imaginal discs, but develops from special imaginal cells called histoblasts. These cells are present as clusters of small and non-dividing diploid cells in the larval epidermis and are laid down during embryogenesis as derivatives of the embryonic epidermis (Madhavan and Schneiderman et al., 1977; Simcox et al., 1991). There are three major histoblast clusters in each larval abdominal hemisegment: an anterior dorsal nest that produces the abdominal tergite, a posterior dorsal nest that produces the intertergal cuticle, and a ventral nest that produces the sternite and pleural cuticle (Madhavan and Madhavan, 1980). Unlike imaginal disc cells, abdominal histoblasts do not divide during larval stages

and are arrested in the G2 phase of the cell cycle (Garcia-Bellido and Merriam, 1971; Madhavan and Schneiderman, 1977). The histoblasts increase in volume about 60-fold during larval stages. At the onset of metamorphosis, histoblasts divide rapidly and replace the adjacent degenerating, polyploid larval epithelial cells (LECs) (Ninov et al., 2007). Ecdysone signaling is required for both proliferation of histoblasts and cell death of LECs (Ninov et al., 2007). The LECs express various segment-polarity genes like *en*, *hh*, *wg*, and *patched* (*ptc*) (Struhl et al., 1997). The expression patterns of these genes in LECs are largely unchanged from those in the mature embryo (reviewed by Hooper and Scott, 1992). Likewise, in the larval epidermis *en* and *hh* are expressed in cells of the posterior (P) compartments (Fig.1; Hama et al., 1990), *ptc* is expressed in cells of the anterior (A) compartments, and *wg* is expressed in cells of the A compartments that are immediately adjacent to the compartment boundary (Fig. 1; Struhl et al., 1997). As in LECs, *en* and *hh* are expressed in all histoblasts of the posterior dorsal nests and in posterior subsets of ventral histoblast nests that later produce the P compartments of the adult abdominal segments. *ptc* expression is observed only in histoblasts of the A compartment that are immediately next to the compartment boundary (Struhl et al., 1997). *wg* is expressed in the same histoblasts as *ptc* and, in addition, in a subset of histoblasts that are located just anterior to the *ptc* expressing cells (Struhl et al., 1997).

The *paired* (*prd*) gene, initially characterized as a pair-rule segmentation gene (Nüsslein-Volhard and Wieschaus, 1980), encodes a transcription factor that contains two DNA binding domains, a paired domain (PD) and an extended *prd*-type homeodomain (HD), in its N-terminal half and a 21-amino acid His-Pro (or PRD) repeat near its C-terminal end that serves as an activation domain (Bopp et al., 1986; Frigerio et al, 1986). In addition to its embryonic segmentation function, *prd* is required for post-embryonic viability and male fertility (Xue and Noll, 1996; Xue et al., 2001; Xue and Noll, 2002). Rescue experiments using the *prd* evolutionary alleles, *prd*-Gsb and *prd*-Pax3, uncovered a novel function of *prd* in the adult cuticle segmentation (Xue and Noll, 1996). Gsb and Pax3 proteins, when expressed under the control of the complete *prd cis*-regulatory region, are able to rescue the embryonic cuticle function of *prd* at low efficiency. Gsb but not Pax3 is able to rescue 10% of *prd* mutants to adulthood when it is present in two copies (Xue and Noll, 1996; Xue et al., 2001). Interestingly, one copy of *prd*-Pax3 enhances the rescue of *prd* mutants to adulthood by *prd*-Gsb (Xue and Noll, 1996). Many of the rescued flies exhibit a segmental phenotype in the abdomen, which exhibits poorly developed and often fused segments (Xue and Noll,

1996). Here we investigated the adult cuticle function using transgenes of *prd* regulated by an incomplete set of *prd* enhancers. One copy of the *prd*Res transgene, which lacks almost the entire downstream *cis*-regulatory region, rescues only 20% of *prd* mutants to adulthood, and most of the rescued flies exhibit partial fusions of abdominal segments. Two copies of *prd*Res rescue this *prd* function to almost wild-type. One copy each of *prd*-mf5, which is under control of the entire downstream region of *prd*, and of *prd*Res rescue the adult cuticle phenotype completely. Conditional knockout of *prd* during post-embryonic stages showed a severe disorganization of the abdominal segments. We could also show that histoblast specific knockout of *prd* is sufficient to produce the most severe abdominal segmental phenotype. These results demonstrate a crucial role of *prd* in the development of adult abdominal segments.

## Results

### *prd* is required for the proper segmentation of the adult abdomen

Our previous results showed that *prd* mutants rescued by the evolutionary alleles of *prd*, *prd*-Gsb and *prd*-Pax3, display an adult abdominal segmentation phenotype (Xue and Noll, 1996). The *prd*-Gsb transgene is able to substitute for all functions of *prd*, though not at the same efficiency as Prd. Unlike Gsb, Pax3 is unable to substitute for all functions of Prd (Xue and Noll, 1996; Xue et al., 2001). However, *prd*-Pax3 enhances the rescue efficiency to adulthood of the *prd*-Gsb transgene (Xue and Noll, 1996). These results indicated that *prd* is required for the proper segmentation of the adult abdomen. Like the post-embryonic viability function of *prd*, the adult cuticle function is not fully conserved in its evolutionary alleles (Xue and Noll, 1996; Xue et al, 2001).

Rescue of *prd* mutants by one copy of *prd*Res, a *prd* transgene that lacks almost the entire downstream region of *prd* (Fig. 2), uncovered a haplo-insufficiency of this transgene. One copy of *prd*Res rescues only 20% of *prd* mutants to adulthood (Table 1), while 80% die during larval stages. Most of the *prd* mutants rescued to adulthood by one copy of *prd*Res show partial fusion of abdominal segments (Fig. 3B, C). Two copies of *prd*Res rescue all *prd* null mutants to adulthood as well as the adult segmentation phenotype of most $prd^-$ flies (Table 1; Fig. 3E, F). One copy each of *prd*Res and *prd*-mf5 (Fig. 2) completely rescues

survival to adulthood and the *prd* mutant adult segmentation phenotype (Table 1; Fig. 3D), which is undistinguishable from wild-type (Fig. 3A). These results suggest that the *prd*Res transgene is haplo-insufficient not only for the survival function, but also for the adult abdominal segmentation function of *prd*. The downstream enhancers are partially redundant for both of these functions and rescue the haplo-insufficiency of the upstream enhancers of *prd*. Finally, we could locate the enhancer for the adult cuticle segmentation in the downstream region to a 1.5 kb *Xba*I-*Pst*I fragment (*prd*-mf2, Fig. 5 of chapter 2) that includes the survival enhancer but not the embryonic stripe enhancer of the downstream region (Fig. 15 of chapter 2).

**Crucial role of *prd* in segmentation of adult abdominal cuticle is confirmed by conditional knockout of *prd***

The partial rescue of *prd* mutants revealed a novel function of *prd* in the segmentation of the adult abdominal cuticle. The abdominal segments are only partially fused in these rescued flies (Fig. 3B, C), while a small fraction of the rescued animals show even properly developed segments (Table 1). This weak adult cuticle phenotype might result from partial rescue of this function by one copy of the *prd*Res transgene. In support of this supposition, two copies of *prd*Res rescue this function almost to wild-type (Table 1; Fig. 3E, F). Therefore, we investigated the adult cuticle function of *prd* in the absence of any functional Prd protein. All known *prd* alleles are embryonic lethal since they were obatined by a screen for defects in larval segmentation (Nüsslein-Volhard and Wieschaus, 1980), and hence cannot be used for this purpose. To rescue the early function(s) of *prd*, we set up a conditional rescue system by which *prd* expression can be silenced after completion of embryonic development.

To this end, we constructed a *prd* transgene (*prd*FRT) that is similar to the *prd*-SN20 transgene but can be inactivated by heat-activated flipase that removes the second exon of *prd*, which is flanked by FRT sites (Fig. 2). One copy of the *prd*FRT transgene is sufficient to rescue all *prd* mutants to adulthood (data not shown), which suggests that the inserted FRT sites do not interfere with the function of *prd*. Upon heat induction of the flipase, the *prd* coding sequence between the two FRT sites is deleted, thus generating a truncated *prd* gene that encodes a non-functional peptide without DNA-binding domain (Frigerio et al., 1986). The flipase expression is controlled temporally through heat shock, which allows us to knockout *prd* during post-embryonic stages. The heat shock was applied at 37°C for an hour,

starting from early second instar, and this step was repeated every 12 hours until eclosion of adult flies. All *prd* mutants carrying the conditional *prd* and heat-inducible flipase transgenes survived to adulthood, which indicates that the heat-shock regime does not affect the viability function of *prd* (data not shown). The rescued *prd* mutants show highly disorganized abdominal segments (Fig. 4B, C) as compared to wild-type flies (Fig. 4A) and even *prd* mutants rescued by one copy of *prd*Res (Fig. 3B, C). The abdominal segments are poorly developed, and the fusion of neighboring epithelial cells within segments is severely compromised (Fig. 4B and C). However, the thorax region is not affected in these rescued animals, which indicates that *prd* is required specifically in the precursor cells of the adult abdominal segments but not in the wing imaginal discs (Fig. 4B). Analysis by scanning electron microscopy revealed that the abdominal segments are fused with each other and that the polarity of the bristles in the abdominal segments is severely affected in these flies (Fig. 5B) when compared to wild type (Fig. 5A).

**Rescue of adult abdominal segmentation phenotype generated by heat shock-induced knockout of *prd***

Conditional knockout of *prd* during post-embryonic stages showed a severe disorganization of abdominal segments in the adult (Fig. 4B, C). To test whether this phenotype is caused exclusively by the loss of the *prd* function rather than the heat-shock regime used to inactivate the *prd* transgene, complete or partial *prd* transgenes were used to rescue the adult segmentation phenotype induced by heat-inactivation of the *prd* conditional-rescue transgene, *prd*-FRT (Fig. 2). One copy of *prd*-SN20 rescues the segmentation phenotype completely, which demonstrates that proper segmentation of the adult abdomen specifically depends on the *prd* function and does not result from the heat-shock regime (Fig. 6A). The segmental phenotype is only partially rescued by one copy of *prd*Res (Fig. 6B, C), which indicates that the flip-out is efficient in this tissue, as the mutant phenotype looks similar to that obtained in the absence of *prd*-FRT (Fig. 3B, C). By contrast, the *prd*-mf5 transgene does not appear to rescue the adult segmentation phenotype produced by the inactivation of *prd*-FRT (cf. Fig. 6D with Fig. 4B, C). Hence the intron and the complete downstream region of *prd*-mf5 cannot contribute much to correct adult segmentation, unless combined with *prd* expression and functions directed by the upstream region and intron of *prd*Res, as observed in a *prd* mutant background (Fig. 3D). But in *prd* mutants the contribution of *prd*-mf5 alone could not be tested for postembryonal functions because these animals die already as unhatched first instar larvae with an only slightly rescued pair-rule phenotype (Fig. 8 of chapter 2). It is

likely that the rescuing expression of *prd*-mf5 depends on the expression of *prd*Res in the developing abdominal segments. This again suggests a similar autoregulation of *prd* expression as observed for the embryonal cuticular and survival functions.

**Adult abdominal segmentation phenotype generated by inactivation of *prd* in histoblasts**

The cells destined to form the cuticle of the adult abdomen, the histoblasts, form clusters of small, non-dividing diploid cells in the larval epidermis (Struhl et al., 1997). The histoblasts only increase in volume and do not divide during larval stages. Upon pupariation, histoblasts begin to divide rapidly and fuse to form the adult abdominal segments, thus replacing the LECs (Lawrence et al., 2004; Ninov et al., 2007).

Because of their role in the formation of the abdominal integument, we investigated whether *prd* is required in histoblasts for proper development of the abdominal segments. To this end, we inactivated *prd* specifically in the histoblasts, taking advantage of a *Gal4* enhancer-trap line, *NP5130-Gal4*, driving UAS-FLP (Ninov et al., 2007). In this line, Gal4 is inserted in the *escargot (esg)* locus, which is expressed in histoblasts, salivary glands, and wing discs and widely used as a histoblast marker (Ninov et al., 2007). Thus, the flipase expressed under the control of *NP5130-Gal4* inactivates the *prd*-FRT transgene in the histoblasts (Fig. 2). This histoblast-specific inactivation of *prd* resulted in the disorganization of the adult abdominal segments (Fig. 7A-C). One copy of *prd*-SN20 transgene completely rescued this abdominal *prd* mutant phenotype (Fig. 7D). Therefore, *prd* is required in histoblasts to control the development of the adult abdominal segments. We speculate that *prd* controls the proliferation or fusion of the histoblasts.

## Discussion

We have shown here that *prd* function is necessary for proper segmentation of the adult abdomen. The conditional knockout of *prd* during post-embryonic stages revealed a crucial role of *prd* in the development of the adult abdominal segments (Fig. 4B, C). The knockout of *prd* in the histoblasts produces a severe disorganization of abdominal segments, which demonstrates that development of abdominal segments depends on *prd* function in histoblasts (Fig. 7A-C). It was gratifying to observe the strong adult segmentation and cuticle phenotype

in animals where the *prd* gene was removed in developing abdominal segments (Figs. 4 and 7), which convincingly showed that the much weaker phenotypes observed in *prd* flies rescued by either transgenes lacking downstream enhancers (Fig. 3) or "*prd* evolutionary alleles" are due to the partial rescue of the adult segmentation function (Xue and Noll, 1996). Although the adult segmentation phenotypes of heat-shock-induced conditional knockouts of *prd* are stronger compared to the phenotypes of partially rescued *prd* flies (Fig. 4B, C), it was not clear whether the complete lack of *prd* function in developing abdominal segments would produce even a stronger phenotype. However, the fact that the abdominal segmentation phenotypes produced by the histoblast-specific knockout of *prd* by the use of *NP5130 Gal4* (Fig. 7A-C), an enhancer trap line expressed throughout the development of histoblasts, are similar to the conditional *prd* knockout phenotypes demonstrated that the heat shock-induced conditional knockout removes most, if not all, of the *prd* function.

Severe defects in the development of abdominal segments and loss of bristle polarity (Fig. 5B) suggests that Prd plays a dual role in adult cuticle development, an early function that is required for the proper development of segments and a late function required for the establishment of proper bristle polarity in these segments. Such a dual role for *prd* has been extensively studied in accessory gland development and function where the early function controls cell proliferation and the late function regulates the expression of various accessory gland genes whose products are transferred to female during copulation (Xue and Noll, 2002). Similar defects in bristle polarity in adult abdominal segments were observed in clones that are mutant for segment-polarity genes like *hh*, *wg*, and *en* (Shirras et al., 1996; Struhl et al, 1997; Lawrence et al., 1999). Since *prd* is known to regulate the expression of *en* and *wg* during embryonic segmentation (Xue and Noll, 1996; Xue et al., 2001), it is likely that *prd* controls the bristle polarity by regulating the expression of some of these segment-polarity genes in the developing abdominal segments. The segment-polarity mutants exhibit normally developed segments except that the bristle polarity is lost, which suggest that the segment-polarity genes are dispensable for the proliferation and fusion of histoblasts (Shirras et al., 1996; Struhl et al, 1997). These observations indicate that there must be other genes regulated by *prd,* which contribute to the severe adult segmentation phenotypes of *prd*. Recently, it has been shown that edcysone signaling is required for the proliferation of histoblasts during pupal stages (Ninov et al., 2007). The adult segmentation phenotypes produced by the removal of *prd* function in histoblasts suggest that *prd* might be controlling the proliferation of histoblasts through edcysone signaling or by directly regulating the

expression of genes involved in cell-cycle progression such as *cycE*. The loss of *prd* affects the development of the dorsal but not of the ventral cuticle in the abdominal segments, which suggests that the *prd* function is required for the proliferation and/or fusion of the anterior and posterior dorsal histoblasts, which form the dorsal cuticle of the abdominal segments in the adult fly.

As observed for the survival function, the upstream enhancers together with the intron are haplo-insufficient for the early function in adult cuticle segmentation that might regulates the proliferation of histoblasts (Table 1; Fig. 3B, C), but not for the late function that controls the orientation of bristles (Fig. 3B, C). The downstream enhancers together with the intron in *prd*-mf5 rescue the haplo-insufficiency of the upstream enhancers for the survival function as well the adult cuticle segmentation (Table 1; Fig 3D). The enhancers in the downstream region controlling the adult cuticle function and the survival of *prd* mutants to adulthood overlap completely (*prd*-mf2; cf. Figs. 5 and 15 of chapter 2). Investigation of sequences controlling adult segmentation in the upstream region would further help us to understand the role of *prd* in adult cuticle segmentation in more detail. The downstream enhancers alone are not sufficient to rescue the adult cuticle function of *prd* (Fig. 6D), but enhance the rescue efficiency of the upstream enhancers (Fig. 3D), which may indicate that the downstream enhancers are dependent on expression activated by the upstream enhancers and might be subject to autoregulation as we observed for embryonic segmentation and survival functions. Unlike the survival and the adult segmentation functions, the larval cuticular function is completely rescued by one copy of the *prd*Res transgene (Fig. 3C of chapter 2). Interestingly, the male fertility function of *prd* absolutely requires the presence of both the upstream and downstream enhancers in *cis* or in *trans* (cf. chapter 2). It is noteworthy that although these enhancer regions cooperate to rescue functions of *prd* they need not to be present in *cis* to each other like in the wild-type gene. They are equally efficient in *trans* since the combination of *prd*Res and *prd*-mf5.4 (line 4 of *prd*-mf5) transgenes, two independent insertions on the third chromosome, rescues all known functions of *prd* as efficiently as one copy of the *prd*-SN20 transgene.

*prd* mutants rescued by the evolutionary alleles of *prd*, *prd*-Gsb and *prd*-Pax3, displayed a partial fusion of abdominal segments, which indicated that the adult cuticle function of *prd* is not fully conserved in its evolutionary alleles (Xue and Noll, 1996). Both *prd*-Gsb and *prd*-Pax3 can rescue the embryonic segmentation function, but only *prd*-Gsb is

able to rescue about 10% of *prd* mutants to adulthood when present in two copies (Xue and Noll, 1996; Xue et al., 2001). Interestingly, the addition of *prd*-Pax3 enhances the survival of *prd* mutants by the *prd*-Gsb transgene (Xue and Noll, 1996). These results suggest that at least two independent functions of *prd* are required for viability, one of which Pax3 is able to perform better than Gsb. Yet Pax3 alone cannot fully substitute for one of the viability functions and therefore cannot rescue *prd* mutants to adulthood (Xue et al., 2001). Moreover, Pax3 rescues the adult cuticle function better than Gsb (Xue and Noll, 1996). Our previous work demonstrated that a chimeric transgene, *prd*-GsbN+PrdC, in which the N-terminal moiety of *prd* is replaced by that of Gsb, rescues all *prd* mutants to adults with a wild-type cuticle (Xue et al., 2001). These results indicate that the survival and the adult cuticle functions of *prd* require the C-terminal moiety of the Prd protein that is significantly different from that of its evolutionary alleles. The motif required for cuticle formation is present in both Gsb and Pax3, the viability function only poorly conserved in Gsb.

## Materials and Methods

### Fly stocks

+; $prd^{2.45}$ *b*; *P*{$ry^{+}$; *prd*-SN20} $ry^{506}$ (Gutjahr et al., 1994),

*y w*; $prd^{2.45}$ *b/CyO*; *P*{$w^{+}$; *prd*Res} (Bertuccioli et al., 1996),

*y w*; $prd^{2.45}$ *b/CyO*; *P*{$w^{+}$; *prd*-mf5.4.*Gal4*} (Xue and Noll, 2002),

$w^{1118}$; $prd^{2.45}$ *b/CyO*; *P*{$w^{+}$; *prd*-mf5.4.*Gal4*} (Xue and Noll, 2002),

*y w*; $prd^{32.12}$/*CyO*; *P*{$w^{+}$; *prd*Res} (Bertuccioli et al., 1996),

*y w*; $prd^{2.45}$ *b P*{$w^{+}$; *prd*-FRT1.3}/*CyO* (Kühn Georgijevic, 2006),

*P{hsFLP}1*, $w^{1118}$; $Adv^{1}$/*CyO* (stock BL-06 from Bloomington stock center; $Adv^{1}$ is a dominant allele of *engrailed* sometimes abbreviated also as $en^{Adv\text{-}1}$),

*y w hs.FLP*; $prd^{2.45}$ *b/CyO* (Kühn Georgijevic, 2006),

+; $prd^{2.45}$ *b/CyO*; $ry^{506}$ (Noll stock-458),

*y w UAS-FLP*; *Sp*/*SM5a*; *MKRS/TM6B* (gift from Konrad Basler), and

*y w*; *P*{*GawB*}*NP5130/CyO* (*esg-Gal4* from DGRC, Kyoto, Japan).

**Rescue of *prd* mutants**

Transheterozygous combination of two *prd* null alleles, $prd^{2.45}$ and $prd^{32.12}$, were used to rescue *prd* mutants to adulthood. Transgenic $prd^{-}$ animals carrying one or two copies of the specified transgenes were obtained as follows. Two types of stocks were established for all transgenes, namely $prd^{2.45}$/*CyO*; P/P and $prd^{32.12}$/*CyO*; P/P (P stands for the P elements that contain the transgenes). To rescue the adult cuticle phenotype of Prd with one or two copies of the transgenes, $prd^{2.45}$/*CyO*; P/P were crossed to $prd^{32.12}$/*CyO* or $prd^{2.45}$/*CyO*; P/P and $prd^{32.12}$/*CyO*; P/P.

**Conditional knockout of *prd***

*prd*-FRT, a conditional *prd* rescue transgene in which the second exon of *prd* is flanked by two FRT sites (Kühn Georgijevic, 2006), was used to knockout *prd* during post-embryonic stages. For this purpose, *y w*; $prd^{2.45}$ *b prd*-FRT1.3 males were crossed to *y w hs.FLP*; $prd^{2.45}$ *b*/*CyO* virgins. After egg-laying for 12 hours 25°C, the parents were removed and transferred to new tubes. The embryos were allowed to develop until the end of the first-instar stage at 25°C and subjected to a heat shock at 37°C for an hour. The heat shock was repeated every 12 hours until eclosion of flies. To knockout *prd* in histoblasts, *y w UAS-FLP*; $prd^{2.45}$ *b NP5130-Gal4/CyO* animals were crossed to *y w*; $prd^{2.45}$ *b prd*-FRT1.3 animals. The eclosed flies were transferred to new tubes and their abdominal phenotypes were analyzed after two days. The pictures were taken using a Zeiss Stemi 2000-C microscope.

**Scanning electron microscopy**

Flies were treated with ether vapors for 5 minutes and taken to a JEOL JSM 6360 LV scanning electron microscope. Flies were kept in the vaccum chamber for a minute, upon which pictures were taken.

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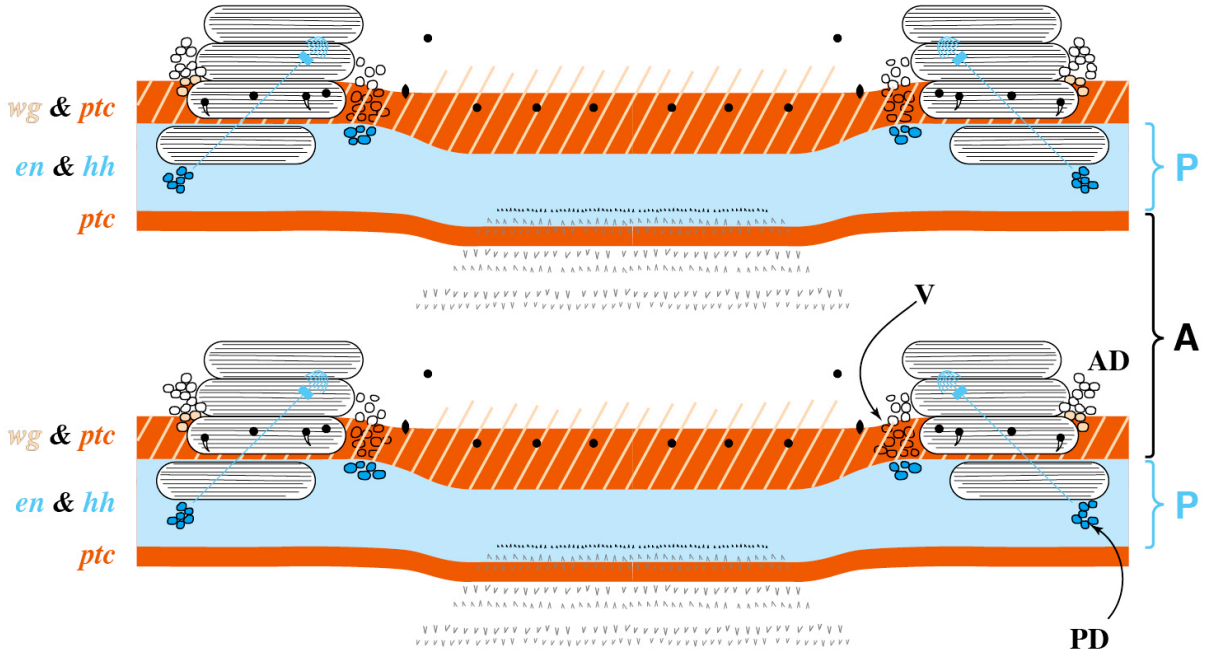


**Figure 1. Expression of segment-polarity genes in the ventral epidermis of the third-instar larva**. The domains of *en* and *hh* expression, which define the P compartment, are shown in blue, those of *wg* and *ptc* in pale orange (hatched) and deep orange, respectively. There are three histoblast clusters in each hemisegment, which will form the adult epidermis. These are the ventral (V), anterodorsal (AD), and posterodorsal (PD) histoblast nests; *en*, *hh*, *wg* and *ptc* are expressed in these cells, as indicated by the color. Also shown are the patterns of ventral hairs (denticles) secreted by the larval cells, sensilla (black symbols) and muscle groups, all of which serve as landmarks. From Struhl et al., 1997.

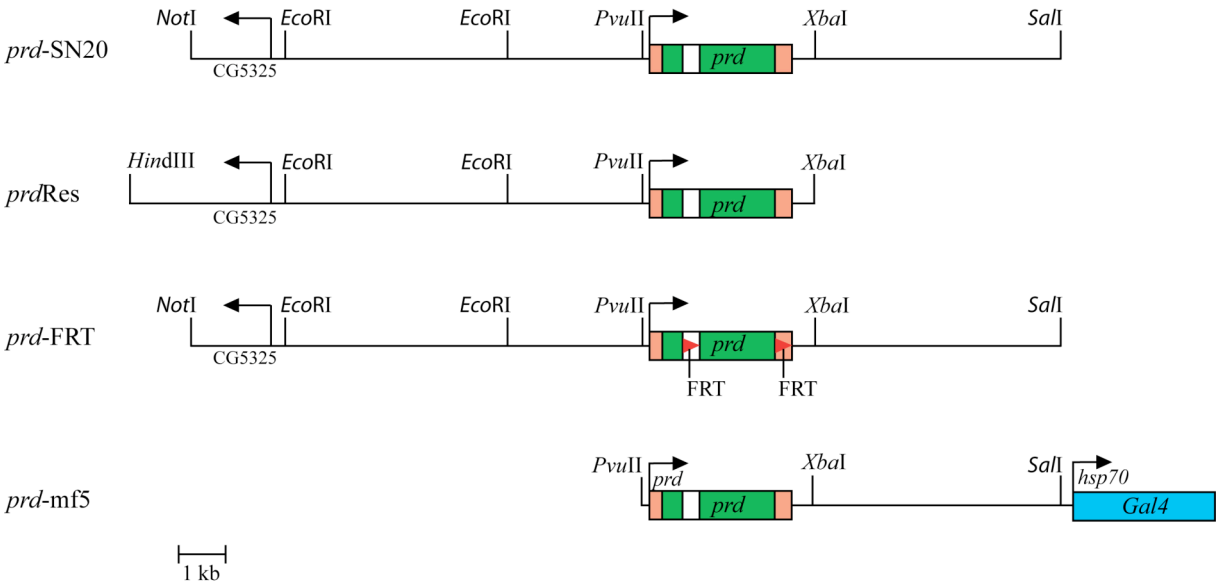


**Figure 2. Map of *prd* transgenes used to rescue abdominal segmentation of *prd*⁻ adults.** The *prd*-SN20 transgene is an 18-kb genomic fragment that includes the entire *prd* gene, consisting of its transcribed region as well as as of its adjacent 9.7 kb upstream and 5.7 kb downstream regions. The *prd*Res transgene includes 11 kb of the upstream, but only 0.5 kb of the downstream region. *prd*-FRT is a *prd* transgene used for conditional rescue of *prd* mutants, in which the second exon of *prd* is flanked by FRT sites and which includes the complete *cis*-regulatory region of *prd* like *prd*-SN20. *prd*-mf5 is a transgene consisting of a genomic fragment of *prd*, extending from the *prd* promoter to the end of the downstream enhancers, that is placed upstream of the *hsp70* basal promoter and the *Gal4* gene (Xue amd Noll, 2002).


**Figure 3. Abdominal segmentation phenotype of *prd*⁻ adults rescued by one or two copies of *prd*Res or one copy each of *prd*Res and *prd*-mf5**. Dorsal views of wild-type (A), *y w*/+; $prd^{2.45}/prd^{32.12}$; *prd*Res/$ry^{506}$ (B, C), *y w*/$w^{1118}$; $prd^{2.45}/prd^{32.12}$; *prd*Res/*prd*-mf5 (D), and *y w*; $prd^{2.45}/prd^{32.12}$; *prd*Res (E, F) females, displaying a wild-type (A, D, E) or mutant phenotype of partially fused abdominal segments. Pictures of 2-day old females were taken under a Zeiss Stemi 2000-C microscope.

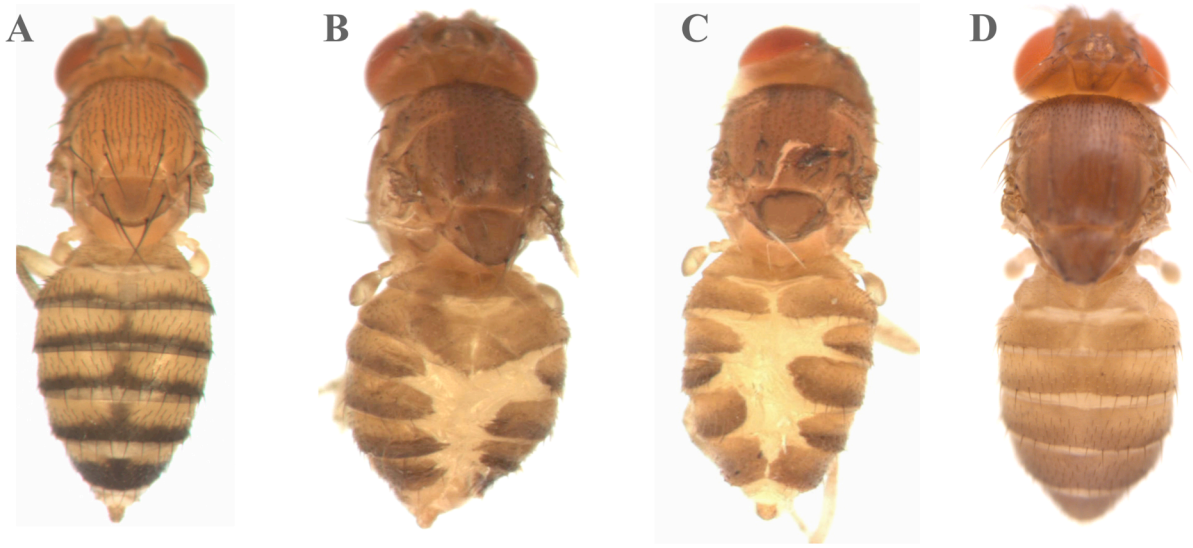


**Figure 4. Abdominal segmentation phenotype of adults generated by conditional knockout of *prd*.** Dorsal views of wild-type (A) and *y w hs-flp/y w*; $prd^{2.45}$ *b prd*-FRT/$prd^{2.45}$ *b* females subjected to a repetitive heat-shock regime during laval and pupal development (B, C) or kept at 18°C througout development (D) show severe defects in the abdominal segments in the absence of a functional *prd* gene (B, C). For details of the heat-shock regime, see Materials and Methods. Pictures of 2-day old females were taken under a Zeiss Stemi 2000-C microscope.

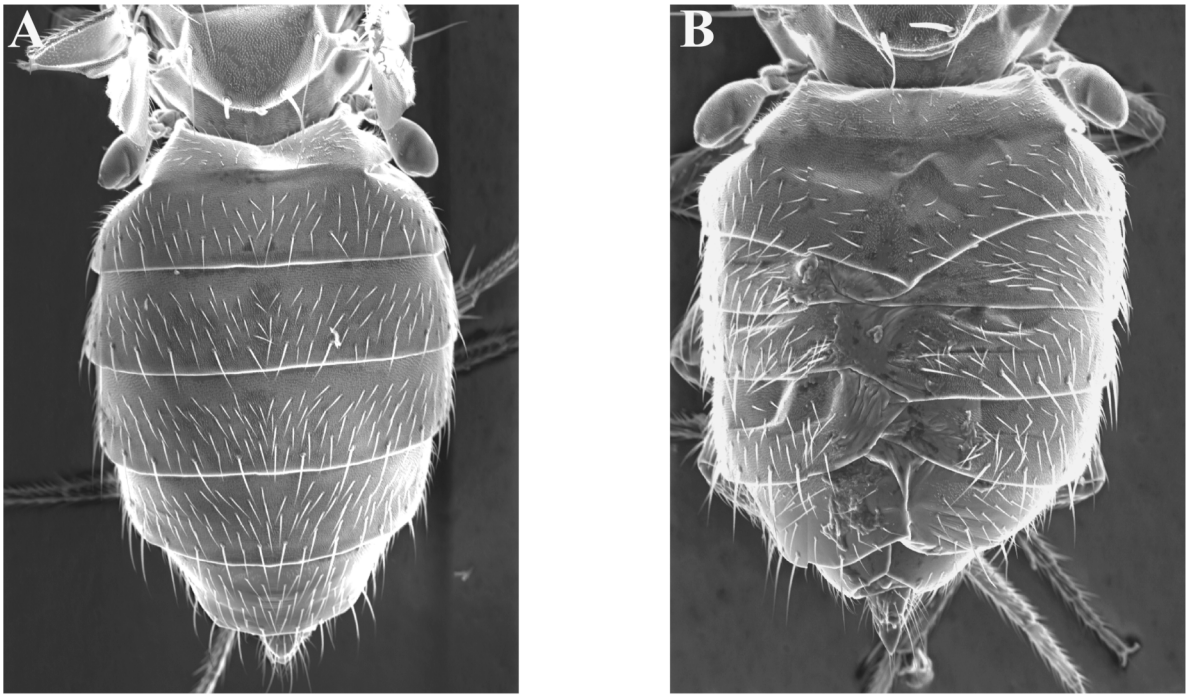


**Figure 5. Loss of *prd* function in the abdominal segments show defects in segmental organization and bristle polarity.** Scanning electron micrographs of abdomen of 2-day old wild-type (A) and *y w hs-flp/y w*; $prd^{2.45}$ *b prd*-FRT/$prd^{2.45}$ *b* females subjected to a repetitive heat-shock regime during laval and pupal development (B) display segmental fusion and loss of bristle polarity in abdominal segments when *prd* is inactivated during post-embryonic stages.

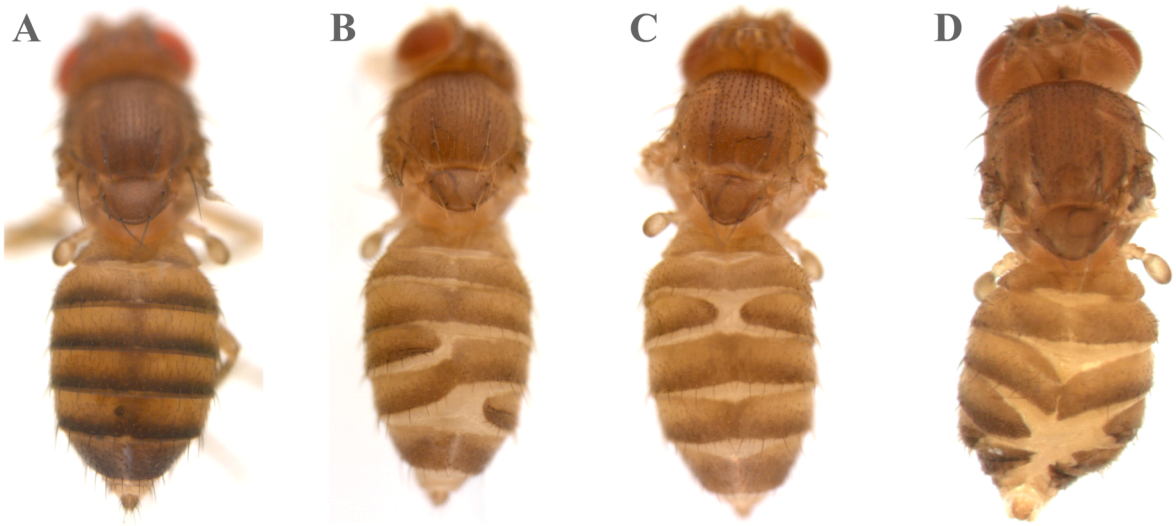


**Figure 6. Rescue of heat-shock induced *prd* knockout phenotype in adult segmentation.** Dorsal views of *y w hs-flp/+*; $prd^{2.45}$ *b prd*-FRT/$prd^{2.45}$ *b*; *prd*-SN20/+ (A), *y w hs-flp/y w*; $prd^{2.45}$ *b prd*-FRT/$prd^{2.45}$ *b*; *prd*Res/+ (B, C), and *y w hs-flp/y w*; $prd^{2.45}$ *b prd*-FRT/$prd^{2.45}$ *b*; *prd*-mf5/+ (D) females that have been subjected to a repetitive heat-shock regime during laval and pupal development show full (A), partial (B, C), and no rescue of the abdominal segmentation phenotype. Pictures of two 2-day old females were taken under a Zeiss Stemi 2000-C microscope.

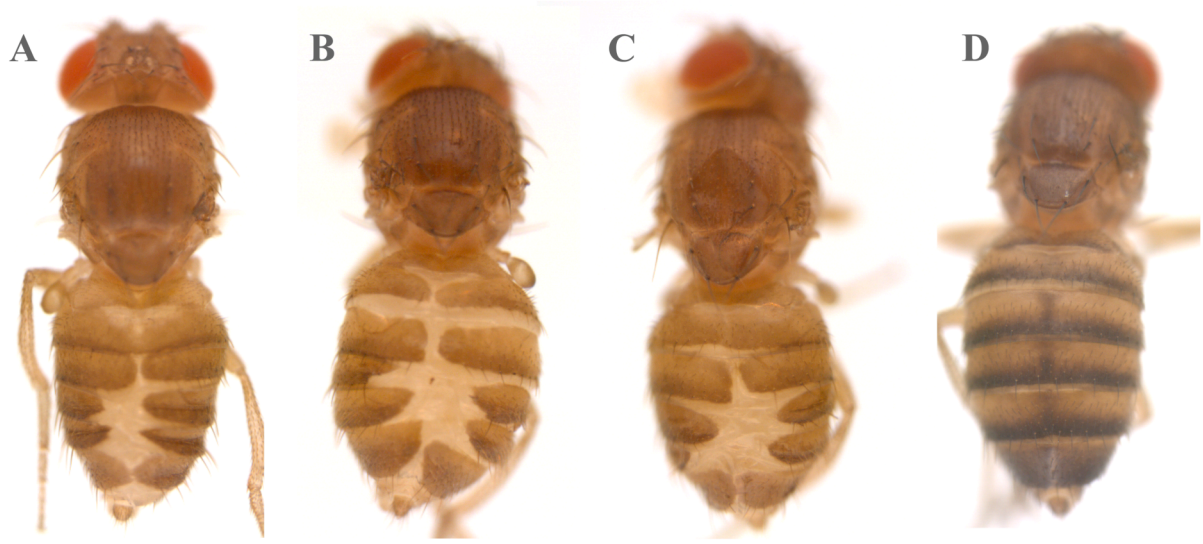


**Figure 7. Histoblast-specific knockout of *prd* produces the adult abdominal cuticular phenotype.** (A-C) Dorsal views of *y w UAS-FLP*/*y w*; $prd^{2.45}$ *b prd*-FRT/$prd^{2.45}$ *b NP5130-Gal4* females display a severe disorganization of abdominal segments at 25°C (A-C), which is rescued completely in *y w UAS-FLP*/+; $prd^{2.45}$ *b prd*-FRT/$prd^{2.45}$ *b NP5130-Gal4*; *prd*-SN20/+ females (D) by *prd*-SN20 at 25°C. Pictures of 2-day old females were taken under a Zeiss Stemi 2000-C microscope.

| Transgene | Rescue to adulthood | Rescue of segmentation |
|---|---|---|
| $prd^{2.45}/prd^{32.12}$; *prd*Res/+ | **20%** | **11%** |
| $prd^{2.45}/prd^{32.12}$; *prd*Res/*prd*Res | > 95% | 84% |
| $prd^{2.45}/prd^{32.12}$; *prd*Res/*prd*-mf5 | > 95% | 98% |

**Table 1. Rescue of adult abdominal segmentation in *prd* null mutants by *prd* transgenes.** The survival efficiency of *prd* transgenes shown in percentage of $prd^{-}$ embryos rescued to adulthood. The rescue efficiency of adult segmentation shown in percentage of *prd* mutants, rescued to adulthood by one or two copies of *prd* rescue transgenes, displayed wild-type segments. Transgenic flies carrying one or two copies of transgenes were produced by the crosses described in Materials and Methods. $prd^{2.45}$ and $prd^{32.12}$ are the null alleles of *prd*.

## Acknowledgments

It gives me an immense pleasure to express my deep thanks and sincere gratitude to my supervisor Prof. Dr. Markus Noll for his excellent supervision and encouragement throughout this study. Besides being an excellent supervisor, Dr. Noll also showed me his enthusiasm, determination and dedication to science, which has made a deep impression on me. I owe him lots of gratitude for showing me his way of doing research.

I would like to take this opportunity to express my gratitude to all those who helped me to complete this thesis. I express my sincere thanks to Dr. Erich Frei for teaching me fly genetics and for stimulating discussions. I would also like to thank Werner Boll, Michael Daube for technical support. Many thanks go to my fellow students in the lab: Sabarinath Chilaka, Yanrui Jiang, Dimitri Krstic, Shilpi Minocha, Jelena Georgijevic-Kühn, Ivan Ostojic, Haihuai He and Cheng Zhang for their help and useful discussions.

This thesis was carefully read by Dr. Erich Frei and thoroughly edited by Prof. Markus Noll to whom I am very grateful. I thank Erich Frei for translating the "Zusammenfassung".

I thank my thesis committee members, Prof. Konrad Basler and Prof. Leslie Pick, for their valuable suggestions.

I thank people from Prof. Walter Schaffner's and Prof. Bernhard Dichtl's group for sharing the weekly progress and literature seminars, which were very useful for my thesis work.

I close the acknowledgments by dedicating this thesis to my parents, with respect and love.

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